Universidade do Minho
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Continued high prevalence of G2P[4] rotavirus strains in Belgium: vaccine induced selective pressure?

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SUMMARY

Continued high prevalence of G2P[4] rotavirus strains in Belgium: vaccine induced selective pressure?

Group A rotaviruses are the leading cause of severe diarrhoea in children under 5 years of age worldwide, accounting for approximately 600,000 child deaths each year. Two rotavirus vaccines, Rotarix® and RotaTeq®, have been licensed in Belgium, and they have strongly reduced the disease caused by rotavirus infections. However, the rotavirus seasons following Rotarix® introduction in 2006 were marked by a relative increase of G2P[4] rotavirus strain prevalence, when compared with seasons before vaccine introduction.

The goal of the work described in this thesis was to perform an epidemiological study on rotavirus prevalence and genotype distribution in Belgium in the 2009-2010 season, as well as to determine if this relative increase in G2P[4] strains is a vaccine-related event or the result of usual genotype fluctuations. A total of 577 stool samples collected from Belgian paediatric patients between August 2009 and July 2010 were analysed, from which 491 samples were positive for rotavirus and further characterised. The most prevalent rotavirus genotype was G2 (61.9%), followed by G1 (25.3%), G4 (3.7%), G9 (3.3%), G12 (2.9%), G3 (1.8%), and G6 (0.4%). Overall, G1, G3, G4, G9 and G12 were mainly associated with the P[8] genotype, whereas all G2 were exclusively associated with P[4]. The majority of the positive samples came from the north of Belgium, especially from the provinces of Antwerp (43.3%), Flemish Brabant (18.2%) and East-Flanders (17.9%). The 2009-2010 season is now the fourth season after Rotarix® introduction in which we observe a further increase on the relative prevalence of G2P[4] strains, suggesting that vaccination might be one of the factors influencing rotavirus genotype distribution, thereby allowing the emergence of G2P[4] strains. The phylogenetic analysis showed that several lineages of G2P[4] were co-circulating in 2009-2010. Geographic distribution of these lineages revealed no differences in the distribution pattern of the larger clusters; small clusters, however, could only be found in specific locations of Belgium. The results obtained showed that is necessary to monitor the effect of vaccination on predominant genotypes, as well as the influence of different lineages in the efficacy of the vaccines.

RESUMO

Continuada e elevada prevalência de rotavírus da estirpe G2P[4] na Bélgica: pressão selectiva induzida pela vacina?

Os rotavírus do grupo A são a principal causa de diarreia grave em crianças menores de cinco anos de idade, sendo responsáveis por cerca de 600,000 mortes infantis anuais. Na Bélgica, duas vacinas foram licenciadas, Rotarix® e RotaTeq®, as quais diminuíram drasticamente a doença causada pela infecção por rotavírus. No entanto, as épocas de rotavírus após a introdução da Rotarix® em 2006 foram marcadas por um aumento relativo da prevalência de rotavírus da estirpe G2P[4], quando comparado com épocas anteriores ao licenciamento da vacina.

O objectivo do trabalho apresentado nesta dissertação consistiu na realização de um estudo epidemiológico sobre a prevalência e distribuição de genótipos de rotavírus na Bélgica durante a época de 2009-2010, bem como determinar se este aumento de estirpes G2P[4] se encontrava relacionado com a vacina ou se era apenas o resultado de flutuações normais de genótipos. Um total de 577 amostras recolhidas de pacientes pediátricos belgas entre Agosto de 2009 e Julho de 2010 foram analisadas, 491 amostras das quais eram positivas para rotavírus. O genótipo mais prevalente foi o G2 (61.9%), seguido do G1 (25.3%), G4 (3.7%), G9 (3.3%), G12 (2.9%), G3 (1.8%), e G6 (0.4%). Em geral, G1, G3, G4, G9 e G12 encontravam-se associados com o genótipo P[8], enquanto que todos os G2 estavam associados ao P[4]. A maioria das amostras positivas teve origem no norte da Bélgica, especialmente nas províncias de *Antwerp* (43.3%), *Flemish Brabant* (18.2%) e *East-Flanders* (17.9%). Após o licenciamento da Rotarix®, 2009-2010 é agora a quarta época em que se observa um aumento da prevalência relativa de estirpes de G2P[4], o que sugere que a vacinação pode ser um dos factores que influenciam a distribuição dos genótipos de rotavírus, permitindo assim o surgimento de estirpes G2P[4]. A análise filogenética demonstrou que várias linhagens de G2P[4] co-circulavam na época de 2009-2010. A distribuição geográfica destas linhagens não revelou grandes diferenças no padrão de distribuição dos grandes *clusters*; contudo, pequenos *clusters* apenas foram encontrados em localizações específicas da Bélgica. Os resultados obtidos mostram que é necessário monitorizar o efeito da vacinação nos genótipos predominantes, assim como a influência de diferentes linhagens na eficácia das vacinas.

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ABBREVIATIONS

AA/BA	Acrylamide/N,N'-methylene-bis-acrylamide
APS	Ammonium persulfate
BLAST	Basic local alignment search tool
bp	Base pairs
DLP	Double-layered particle
dNTP	Deoxynucleotides
ds	Double stranded
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
EtBr	Ethidium bromide
EU	European Union
FDA	Food and Drug Administration
HRV	Human rotavirus
KDa	Kilo Dalton
MEGA	Molecular Evolutionary Genetics Analysis
MQ-water	Mili-Q water
MW	Molecular Weight
NaAc	Sodium acetate
NSP	Non-structural proteins
NTPase	Nucleoside triphosphatase
ORF	Open reading frame
ORS	Oral rehydration salts
PABP	poly(A)-binding protein
PAGE	Polyacrylamide gel electrophoresis
RER	Rough endoplasmic reticulum
RNA	Ribonucleic acid
RNase	Ribonuclease
RT-PCR	Reverse transcription – polymerase chain reaction
RV	Rotavirus
SLP	Single-layered particle
TBE	Tris-borate-EDTA
TEMED	Tetramethylethylenediamine
TLP	Triple-layered particle
USA	United States of America
UV	Ultraviolet
VP	Viral protein
VTM	Viral transport medium
WHO	World Health Organization

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1. INTRODUCTION

Rotaviruses are the leading cause of severe diarrhoea in infants and young children worldwide, being responsible for more than 600,000 deaths each year, mainly in developing countries (1-4).

1.1. Rotaviruses

1.1.1. Discovery of human rotaviruses

Human rotavirus (HRV) particles, and their association with severe endemic infantile diarrhoea, were first discovered in 1973 by Bishop and colleagues, after visualization of the particles by electron microscopy (EM) in duodenal mucosa biopsies, obtained from infants and young children with acute diarrhoea. Shortly afterward, other researchers were also able to identify HRV particles in faeces, through EM. It soon became apparent that this 70 nm particle was responsible for 35% to 50% of the hospitalizations in children with acute gastroenteritis during the first 2 years of life. In the following years, various investigators reported the detection of rotaviruses (RVs) in faeces of paediatric patients with diarrhoeal illness, and it was soon established that rotaviruses were the most important etiologic agent of diarrhoea in infants and young children, in both developed and developing countries (5, 6).

1.1.2. Virion structure

The name rotavirus is derived from the Latin *rota*, which means wheel, because the virus particle is characterised for having a distinct morphology that resembles a wheel with short spikes and a well-defined, smooth outer rim, when viewed by EM (Figure 1). Rotaviruses are approximately 100 nm in diameter (including spikes), icosahedral, non-enveloped, and they can be observed by EM in three forms: **(i)** the triple-layered particles (TLPs), consisting of a core, an inner capsid and an outer shell; **(ii)** the double-layered particles (DLPs) missing the outer shell, and **(iii)** single-layered particles (SLPs) consisting of the core only (5, 6).

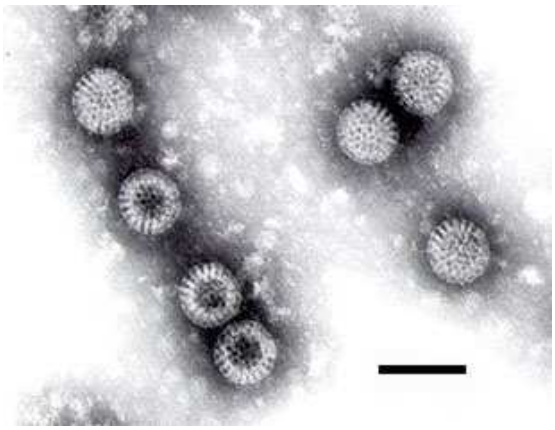

Figure 1: Human rotavirus particles observed by negative stain EM. Bar = 100 nanometers.

Photo Credit: F.P. Williams, U.S. EPA (http://www.epa.gov/microbes/rota.html).

An infectious rotavirus particle (TLP structure), also designated virion, is composed of three concentric protein layers (Figure 2B). The core particle contains the double-stranded (ds) RNA gene segments and enzyme complexes responsible for the processes of RNA transcription and replication, and is mainly composed of VP2, arranged as 60 dimers (120 molecules) surrounding the dsRNA, along with small amounts of VP1 and VP3 (12 molecules each). The middle layer, or inner capsid, is composed of 260 trimers (780 molecules) of VP6. The outer capsid is composed of VP7 (arranged in 260 trimers) and penetrated by 60 dimers of the spike protein VP4, which extend from the smooth surface of the outer shell (5-7).

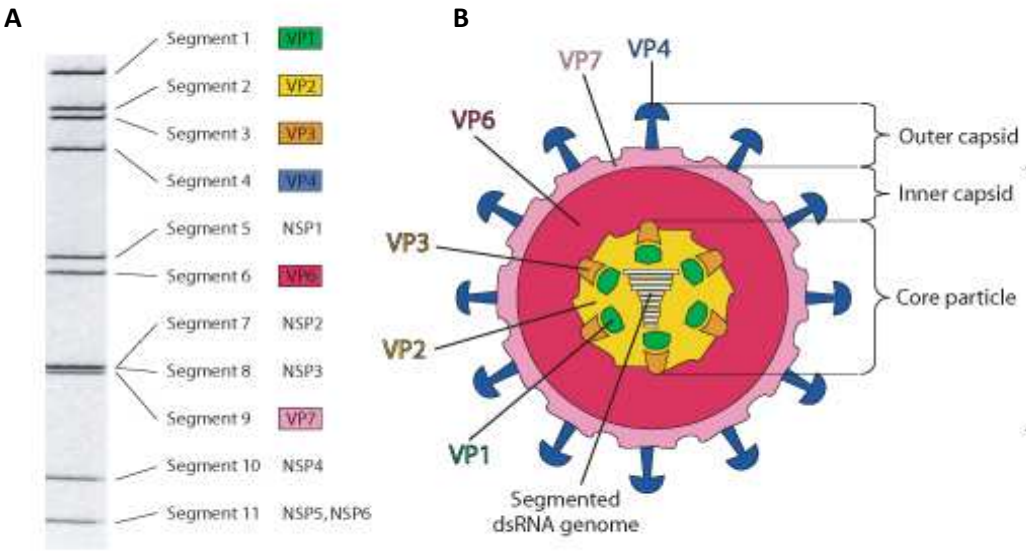

Figure 2: (A) Migration pattern of the rotavirus gene segments separated on a PAGE-gel, and their respective protein products. **(B)** Schematic representation of a mature rotavirus particle (6).

1.1.3. Genome organization

Rotaviruses are the only mammalian agents known to contain a genome of 11 segments of dsRNA, encoding 6 structural viral proteins [VP] (VP1 to VP4, VP6 and VP7) and 6 non-structural viral proteins [NSP] (NSP1 to NSP6). Rotavirus non-structural proteins are found in infected cells but not in mature particles of the virus. Each dsRNA segment encodes only one protein with the exception of the 11th gene segment which encodes two (NSP5 and NSP6) (8-12). The characteristics of these gene segments and their protein products are summarized in Table 1.

Table 1: Rotavirus (strain SA11) gene coding assignments (5, 13, 14).

Gene segment	Length (bp)	Protein	MW (kDa)	Properties
1	3302	VP1	125	RNA-dependent RNA polymerase, RNA binding, interacts with VP2 and VP3.
2	2690	VP2	94	RNA binding, interacts with VP1.
3	2591	VP3	88	Binding activity of mRNA, guanylyl and methyl transferase, interacts with VP1.
4	2362	VP4	86.7	Hemagglutinin, cell attachment protein, neutralization antigen, protease enhanced infectivity, virulence determinant.
5	1611	NSP1	58.6	RNA binding, zinc finger protein, assembly, antagonist of interferon response.
6	1356	VP6	44.8	Interacts with NSP4 at maturation stage.
7	1104	NSP3	34.6	RNA binding, PABP homologue, interacts with eIF4G, inhibits host translation.
8	1059	NSP2	36.7	Important for genome replication/packaging, viroplasm formation, NTPase, RNA binding.
9	1062	VP7	37.4	Glycoprotein, neutralization antigen, involved in cell attachment, calcium-binding protein.

10	751	NSP4	20.2	Transmembrane glycoprotein, viral enterotoxin, modulates intracellular Ca^{2+} levels, role in viral morphogenesis and budding process, virulence determinant due to mutations in the NSP4 gene.
11	667	NSP5	21.7	Phosphoprotein, RNA binding, protein kinase, forms viroplasms with NSP2.
		NSP6	12	Interacts with NSP5.

The rotavirus gene segments range in size from 667 (segment 11) to 3302 base pairs (segment 1), with the total genome containing approximately 18,500 bp (10, 15). Furthermore, group A rotaviruses exhibit a typical electrophoretic migration pattern (Figure 2A) that is composed of four high molecular weight segments (numbered 1 to 4), two middle-sized segments (5 and 6), a distinctive triplet of segments (7 to 9), and two smaller segments (10 and 11). Rotavirus dsRNA segments are base-paired end to end, and the positive-sense strand contains a 5' cap sequence $m^7GpppG^{(m)}GPy$. All the gene segments, except for gene 11, possess only one long open reading frame (ORF) after the first initiation codon, and have conserved sequences at their 5' and 3' ends. Generally, these terminal sequences contain important signals for gene expression, genome replication and assembly of the viral genome segments (5).

1.2. Mechanisms of genomic diversity

There are several mechanisms that contribute for rotavirus evolution and diversity, and they are mainly generated through accumulation of point mutations, genomic reassortment, and genome rearrangements (16-19).

1.2.1. Point mutations

Point mutations are a result of genetic drift due to the error-prone nature of RNA-dependent RNA polymerase. In addition, point mutations accumulate at a high rate, leading to the emergence of antibody escape-mutants, which contributes to RV diversity (20-22).

1.2.2. Genomic reassortment

Genomic reassortment is a rather common event in rotaviruses due to the segmented nature of their genome, resulting in the formation of novel reassortant virus with gene segments from more than one parental strain, which significantly contributes to the overall genetic diversity of RVs through genetic shift (15, 21, 23, 24). Reassortment occurs when a cell is infected with more than one RV strain, resulting in the exchange of segments after co-infection (12). This mechanism is most efficient when the co-infecting viruses are closely related, i.e. belonging to the same group/species, since the percentage of progeny containing novel assortments of gene segments is higher in this case (14, 18).

Animal rotaviruses are a potential reservoir for genetic exchange with HRVs, forming novel rotavirus strains through interspecies transmission. Interspecies transmission may occur either by direct transmission of the virus or by contributing one or several genes to reassortants with a human strain genetic backbone. Zoonotic strains are rarely detected, and only a few HRV strains have high homology to all 11 genes of animal rotaviruses. In contrast, interspecies transmission through reassortment has been documented more often than transfer of the whole virus gene constellation (11, 18-20, 25-28).

1.2.3. Genome rearrangement

Genome rearrangement, which is another example of genetic shift, consists in alterations of considerable sequence fractions within single genome segments, sometimes in the form of deletions, insertions, and/or (partial) duplications (14, 21). Most rearrangements in rotaviruses have been observed in genes coding for non-structural proteins (gene 5, 7, 8, 10 and 11), mainly gene 11, although it was also reported for gene 6 (5, 14, 29).

1.3. Classification of rotaviruses

In 1978, the International Committee on Taxonomy of Viruses (ICTV) officially adopted *Rotavirus* as a genus in the Reoviridae family. Since then, rotavirus strains have been classified using different methods, which are described bellow (10, 24).

1.3.1. Antigenic Specificity

1.3.1.1. Serogrouping

To date, seven serogroups of rotavirus (groups A-G) have been determined based on the antigenic cross-reactivity properties of VP6 (24, 30). Group A rotaviruses are the most important enteric pathogens in humans and in a wide range of mammalian and avian species, due to their high prevalence and pathogenicity in both classes. Groups B and C have been identified in some mammalian species, including humans (8, 30-32). Human group B rotaviruses, in particular, were first detected in China and associated with severe diarrhoea, mainly in adults. Initially, they were just restricted to China, but later they were responsible for sporadic outbreaks in India (since 1998) and Bangladesh (since 2001). Thus far, serogroups D-G have been found mostly in birds, except for group E that can only be found in pigs (5, 24, 30, 33, 34).

1.3.1.2. Subgrouping

Subgrouping is based on the highly immunogenic properties of VP6 since antibodies for this protein are detected immediately after infection. In addition, VP6 bears different epitopes, which allow group A rotaviruses to be further subdivided into four different sub-groups (SG): SG I, SG II, SG I+II and neither SG (non-I non-II), based on the reactivity with subgroup specific monoclonal antibodies (mAb) (1, 14, 35, 36).

1.3.1.3. Serotypes

Serotypes are defined by cross-neutralization assays that measure the reactivity of antibodies to the outer capsid proteins VP4 and VP7 (8, 18). These viral proteins are capable of independently inducing neutralizing antibodies. Consequently, VP7 and VP4 became the basis of a dual classification system defining G serotypes (G for **G**lycoprotein) and P serotypes (P for **P**rotease-sensitive), respectively. Thus far 15 G-serotypes and 15 P-serotypes (P1A, P1B and P2 to P14) have been identified (12, 18, 37). However, because antigenic characterization is time-consuming and proper immunological reagents are not always available, VP7 and VP4 serotyping have been slowly replaced by genotyping (1, 8, 37).

1.3.2. Electropherotyping

By electropherotyping, rotaviruses are classified into two major groups: the long (L) and the short (S) electrophoretic profiles, based on the electrophoretic mobility of gene segments 10 and 11. In the long-electropherotype phenotype, segment 11 is standard-sized, and thus migrates faster than segment 10, whereas the short-electropherotype results from a partial duplication in gene 11, which causes it to migrate more slowly than segment 10. Generally, the short profile exhibits subgroup I specificity and the long profile exhibits subgroup II specificity. In contrast, long profile-subgroup I linkage is mostly found in strains of non-human origin (14, 18, 38, 39).

1.3.3. Genogrouping

Genogrouping is based on the overall similarity of the genomic RNA constellation of HRVs, assessed by RNA-RNA hybridization assays. Three genogroups of HRVs have been established, and they are represented by the reference strains Wa, DS-1 and AU-1. In this classification system, a strain is considered to belong to a certain genogroup if it contains at least seven gene segments similar to those in that particular genogroup (8, 15). The most common HRVs strains belong to the Wa-like and DS-1-like genogroups, which integrate genotypes usually associated with P[8] and genotypes usually associated with P[4], respectively. AU-1–like is a minor genomic constellation that comprises a group of globally circulating but generally rare strains, mainly with the G3P[9] combination (16, 18, 37).

1.3.4. Genotyping

Genotype-specific classification is based on results from reverse transcription-polymerase chain reaction (RT-PCR) performed with type specific primers, followed by nucleotide sequence analysis (3, 40). Through genotyping, group A rotaviruses can be classified by a binary system that recognizes distinct genotypes of VP7, or G-genotypes, and distinct genotypes of VP4, or P-genotypes, based on identities between sequences of cognate rotavirus gene segments. P-genotypes are designated by an Arabic number between square brackets (8, 12, 41). Conventionally, a cut-off value of 89% amino acid sequence identity has been used to define different G and P

genotypes. For VP7, this amino acid identity cut-off value yields an almost complete concordance with the different G serotypes, though for VP4 that does not result in an absolute concordance between different P genotypes and P serotypes (8, 15, 37). Strains that have more than 89% amino acid identity are considered to be of the same genotype (9).

Various combinations of G- and P-genotypes exist and currently 23 G-genotypes and 32 P-genotypes have been described so far, based on the genetic distance between genotypes (16, 40). Nonetheless, most of these genotypes are found in animals, and only a few are known to be responsible for the majority of rotavirus infections in humans. G1P[8], G2P[4], G3P[8], G4P[8], and G9P[8] are the genotype combinations of the most epidemiological importance in humans worldwide (4, 25, 26, 41, 42).

1.3.5. Full genome based classification

Recently, a new classification system was proposed and developed for group A rotaviruses strains, by Matthijnssens and colleagues. This classification system is based on nucleotide identity cut-off percentages that were calculated for all viral proteins in order to distinguish different genotypes for each of the 11 rotavirus gene segments (8, 10, 12, 32). Using this approach, the complete genome of individual RV strains are described using the abbreviations Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx which represent the genotypes of, respectively, VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6, with x indicating the Arabic number of the genotype (8, 24, 25). Up until now, 23 G (Glycosylated, VP7), 32 P (Protease sensitive, VP4), 13 I (Inner capsid protein, VP6), 6 R (RNA polymerase, VP1), 6 C (Core protein, VP2), 7 M (Methyltransferase, VP3), 16 A (Antagonist of interferon, NSP1), 6 N (NTPase, NSP2), 8 T (Translation enhancer, NSP3), 12 E (Enterotoxin, NSP4), and 8 H (Phosphoprotein, NSP5) genotypes have been identified (10, 16).

1.4. Mechanisms of infection

1.4.1. Transmission

Rotaviruses are normally transmitted by the faecal-oral route, and they infect the mature villi of the gastrointestinal tract of infants and young children, causing gastroenteritis. The incubation period of RVs is estimated to be less than 48 hours (5, 31, 42-44).

Rotaviruses are very resistant to physical inactivation and environmental conditions, remaining infectious for a prolonged period of time in several settings. In addition, a large number of viral particles are shed in faeces, and just a small amount is necessary to cause disease, thus contributing to the efficient spread of the HRVs to any susceptible host. Transmission can occur via person-to-person contact and also through other vehicles such as water, fomites (e.g. toilet handles, sinks and other surfaces) and occasionally food (5, 43, 45).

Although RV infection was thought to be restricted to the intestine, recent studies have shown otherwise (46). There have been some reports of antigenemia or viremia in children with rotavirus diarrhoea and also some rare cases involving extraintestinal sites, such as the respiratory tract, liver, kidney, and central nervous system (47-53).

1.4.2. Replication cycle

Following oral ingestion, rotaviruses infect the mature enterocytes on the tips of the small intestinal villi, in which viral replication occurs (54).

The molecular details of rotavirus adsorption, entry and uncoating are complex and not yet completely understood. The first step in the RV infection consists of the virus attachment to the surface of host cells in which, as expected from their locations in the virus structure, VP4 and VP7 play an important role. VP4 is the viral attachment protein that binds the triple-layered particles (virions) of RVs to the cell surface. In the intestinal tract, VP4 is cleaved by proteolytic enzymes in VP8* and VP5*, which enhances viral penetration and infectivity (5, 7, 13, 43, 55). The cellular receptors of rotavirus have not been fully described, but viral penetration is characterised by a sequence of interactions with primary and secondary receptors, the latter acting as co-

receptors in a post-attachment step (7, 43). Several reports have shown that some RV strains initially attach to the sialic acid (SA) residues present in compounds of the cell surface. Besides SA-containing compounds, several integrins and the heat-shock protein 70 (HSP70) were also proposed as mediators of cell entry, acting as co-receptors. Current data suggest that rotaviruses may enter cells by two mechanisms, either by receptor-mediated endocytosis, or direct penetration (5, 7, 13). In both cases, when the virus enters the cell, the outer capsid from TLP is removed by cellular enzymes and a low intracellular Ca^{2+} level, thereby activating the viral RNA polymerase in the VP1-VP2-VP3 transcription complex. The transcriptionally active DLP is at that time released into the cytoplasm of the infected cell, where replication takes place. Large numbers of plus stranded mRNA molecules (5'-capped but not polyadenylated) are then transcribed from all 11 RNA segments by DLP-associated enzymes, exiting the DLP through aqueous channels (5, 7, 13, 43, 56). The new viral mRNA molecules derived from each of the genome segments are either translated to generate the viral proteins encoded by the segment, with their translation products accumulating in the cytoplasm, or serve as templates for genome replication in intra-cytoplasmic inclusion bodies designated "viroplasms" (13, 43).

Non-structural proteins are also involved in various stages. NSP3, for instance, is involved in translation, competing with cellular poly(A)-binding protein (PABP) for interaction with the eukaryotic translation initiation factor eIF4G, and specifically binding the 3'-end of mRNA. NSP2 and NSP5 are responsible for the formation of viroplasm. Besides NSP2 and NSP5, the viroplasm also contains VP1, VP2, VP3, VP6, NSP4 and mRNAs transcribed from all genomic segments (5, 7, 43). The self-assembly of the virus occurs in the viroplasm, forming DLPs containing one of each of the 11 RNA segments. After virus assembly, the DLPs migrate to the rough endoplasmic reticulum (RER) to start the budding process (i.e. acquirement of the virus external envelope), with NSP4 acting as an intracellular receptor for VP6. As a result, the DLPs are transiently enveloped by a RER-derived layer that is lost during the final maturation step when VP7 and VP4 are incorporated. Finally, the mature infectious virus particles (TLPs) are released by cell lysis (7, 13, 43, 56).

1.4.3. Pathophysiology and clinical manifestations

Rotaviruses destroy the enterocytes lining the villi of the small intestine, resulting in the development of extensive cellular necrosis of the epithelium (58). Destruction of the affected cells causes microvillus denudation and shortening, villous atrophy, loss of digestive enzymes and a reduction of absorption of nutrients, which leads to an increased osmotic pressure in the gut lumen, resulting in diarrhoea. Other distinguishable features of RV infection are the presence of enlarged cisternae of the ER, mononuclear infiltration and morphologic intestinal cell alteration from columnar to cuboidal. Crypt cell hyperplasia also occurs in order to compensate for epithelial cell loss, accompanied by increased fluid secretion that contributes to the severity of diarrhoea (43-45, 57, 58). These changes induced by RV infection lead to several clinical manifestations. In general, the main symptoms of rotavirus gastroenteritis are fever, abdominal pain, lethargy, (mild to severe) diarrhoea and vomiting (31, 44). Common complicating features of acute gastroenteritis include hypovolemic shock and dehydration, electrolyte disturbances, metabolic acidosis and nutritional deficiencies. In the worst-case scenario, acute gastroenteritis can even lead to death (31, 44, 45). The symptoms of the disease normally disappear within 3–7 days but may last for 2–3 weeks. Infection can also be asymptomatic, but that is more likely to happen in adults, although it can also occur in reinfected infants (44, 45).

Several viral proteins appear to be implicated in rotavirus pathogenicity. VP4, for example, is thought to be a major determinant of pathogenicity. NSP4 inhibits sodium co-transport mechanisms and produces an increase in intracellular Ca^{2+} concentration, which disturbs cellular electrolyte homeostasis (43, 45, 59). NSP4 is also a viral enterotoxin capable of inducing age-dependent diarrhoea of its own in mice in the absence of histological changes in the intestine. A peptide of NSP4, an active enterotoxin, is released from infected cells and thought to bind to receptors on uninfected cells, activating a signaling pathway that produces diarrhoea (7, 43, 59, 60). By an unknown mechanism, suggested by some investigators to be NSP4-dependent, rotavirus can also increase intestinal motility through stimulation of the enteric nervous system (55, 61).

1.5. Immunity

Epidemiologic studies have suggested that natural rotavirus infection efficiently protects against severe disease associated with reinfection: the first episode of RV gastroenteritis is typically the most severe and subsequent infections are usually less severe or even asymptomatic (62). In addition, symptomatic and asymptomatic infections seem to provide similar protection. Velázquez and colleagues, for example, reported that after the first infection with rotavirus 87% of the children were protected against severe gastroenteritis, whereas the second infection reduced the risk of severe disease by almost 100% (63, 64).

The mechanisms responsible for immunity to RV infections are not entirely understood, but studies in both humans and animal models have enlightened the role of antibodies and the importance of systemic and local immunity (5, 55). RV infections in humans stimulate the production of high levels of anti-rotavirus antibodies of the IgG, IgM, and IgA classes in serum, saliva, and intestinal secretions (65, 66). Primary infection with rotavirus elicits a predominantly serotype-specific, serum-neutralizing antibody response to the virus, therefore, providing homotypic immunity and also partial protection against other serotypes, whereas subsequent infections elicit a broader, heterotypic antibody response (41, 43, 44, 64). Some studies have suggested that high levels of rotavirus-specific serum antibodies provide a good correlate of protection against relatively severe disease (61, 66-68). Other reports, however, have pointed out the role of local intestinal immunity in protection against subsequent RV infections. In fact, these studies revealed that high levels of intestinal rotavirus IgA antibody, produced in the immunologic response occurring at the intestinal mucosal surface, appeared to be the most efficient mechanism of long-term protection against RV infection (61, 62).

The role of cell-mediated immunity in the immune response to rotavirus infection is also under investigation in both humans and animals. Studies have shown that $CD8^+$ T cells seem to mediate virus clearance rather than confer complete immunity to RV reinfection. In addition, $CD4^+$ T cells are also involved in resolution of primary RV infection, particularly helping in the production of rotavirus-specific antibodies (5, 61, 66, 69).

Rotavirus antibodies have also been found in breast milk, however, the precise effects of breastfeeding on the incidence of infantile gastroenteritis remains unclear. Breastfeeding may confer immunity to newborns, but its lasting protective effect appears to be modest (5, 61, 70).

1.6. Diagnosis

Diagnosis of rotavirus infection is relatively easy because large numbers of infectious particles (between 10^{10} and 10^{12}/mL stool) are shed in the stool of infected persons (43, 45). There are various techniques that can be used for detection of RVs in stool specimens. Electron microscopy is not so widely used anymore, except in more comprehensive studies (7). Nowadays, the techniques most frequently used for RV detection are enzyme-linked immunoassays (ELISA) and passive particle agglutination tests (e.g. latex agglutination test), because they are easy to perform, provide rapid results, and are more sensitive than many of the other tests (43, 45). The method of choice in many laboratories is mostly ELISA because, aside from being sensitive, ELISA does not require specialized equipment and has a built-in control for nonspecific reactions. Flow cytometry was also described as another, and possibly even more, sensitive method for RV detection and quantification of infectious rotaviruses (72, 73).

Molecular techniques are highly sensitive and, consequently, they are increasingly being applied for both detection and genotyping of rotaviruses. RT-PCR, the most important of these techniques, is performed with rotavirus specific primers that are complementary to common and type-specific regions of the VP6, VP7 and VP4 genes, thus permitting not only sensitive detection, but also subgroup, G-type and P-type determination, respectively (43, 71, 74).

1.7. Treatment

No specific antiviral therapy is available for the treatment of rotavirus disease (44). However, there are some courses of action that can help reduce the severity of the symptoms. Oral rehydration therapy is a primary tool for fighting dehydration and, since its introduction, various formulations of oral rehydration salts (ORS) with

reduced osmolarity have been shown to be quite effective in treating mildly to moderately dehydration, reducing the mortality rate of children suffering from severe diarrhoeal disease (43, 45, 75, 76). Nevertheless, children who cannot take ORS because of abdominal distention, excessive vomiting, or lethargy, are often treated with intravenous fluids. Severely dehydrated children should also be referred to intravenous hydration (45, 75, 76). Furthermore, oral immunoglobulins seem to affect the duration of diarrhoea and virus shedding but are not routinely used (43).

Zinc deficiency observed in diarrheic children in developing countries has prompted studies regarding the effect of zinc supplementation in children with diarrhoea. Many studies have suggested that zinc supplementation can significantly reduce the severity of diarrhoea and the duration of the episodes and, as a result, WHO is now recommending administration of zinc supplements. Nevertheless, additional studies are necessary to provide insight into the mechanism by which zinc seems to be effective (45, 75, 77, 78).

1.8. Rotavirus Epidemiology

1.8.1. Burden of rotavirus disease

Rotaviruses, in particular, group A rotaviruses, are the leading etiological agents of severe gastroenteritis in infants and young children worldwide, accounting for approximately 39% of child deaths associated with diarrhoea (23, 79-81). Every year, in both developed and developing countries, RVs are estimated to cause more than 125 million infections, 25 million clinic visits, 2 million hospitalizations, and 611,000 deaths (range 454,000-705,000) in children under 5 years of age (1, 15, 41, 55, 82). The majority of these deaths occur in low and middle-income countries. In addition to causing morbidity and mortality in children, RV gastroenteritis imposes a major economic burden on health care systems and families (26, 32, 80, 83).

From a literature review of studies published during 1975–1985, De Zoysa and Feachem concluded that the incidence of rotavirus disease was similar in both developed and developing countries, indicating that improvements in water supply, hygiene, and sanitation were unlikely to prevent disease (84-86). However, poor conditions, malnutrition and higher incidence of infections, which weaken the immune

system, may eventually affect the outcome of RV infections (1, 30). This means that the proportion of severe to mild cases may differ, resulting in more child deaths in low-income countries compared with high-income countries (1).

1.8.1.1. Developing countries: an overview

In developing countries, RVs are a major public health threat for children, causing high morbidity and mortality (Figure 3). Approximately, 85% of rotavirus-associated deaths occur in South Asia and sub-Saharan Africa (5, 87).

Asia has some of the most-populated countries of low-income status, six of which are in the top ten countries with the highest number of rotavirus disease–associated deaths. In fact, 55% of all rotavirus–related deaths occur in this region, being estimated in 1,230,000 deaths for the year 2003 (88). In their two-year period study (2001-2003), the Asian Rotavirus Surveillance Network (ARSN) found that 43% of all Asian children hospitalized with diarrhoea were infected with rotavirus (88, 89). In China, in particular, RVs are responsible for approximately 2.5 million infections, 230,000 hospitalizations and 33,000 child deaths every year (2). In Bangladesh, rotaviruses cause 6,000–14,000 deaths per year in children under 5 years of age (82).

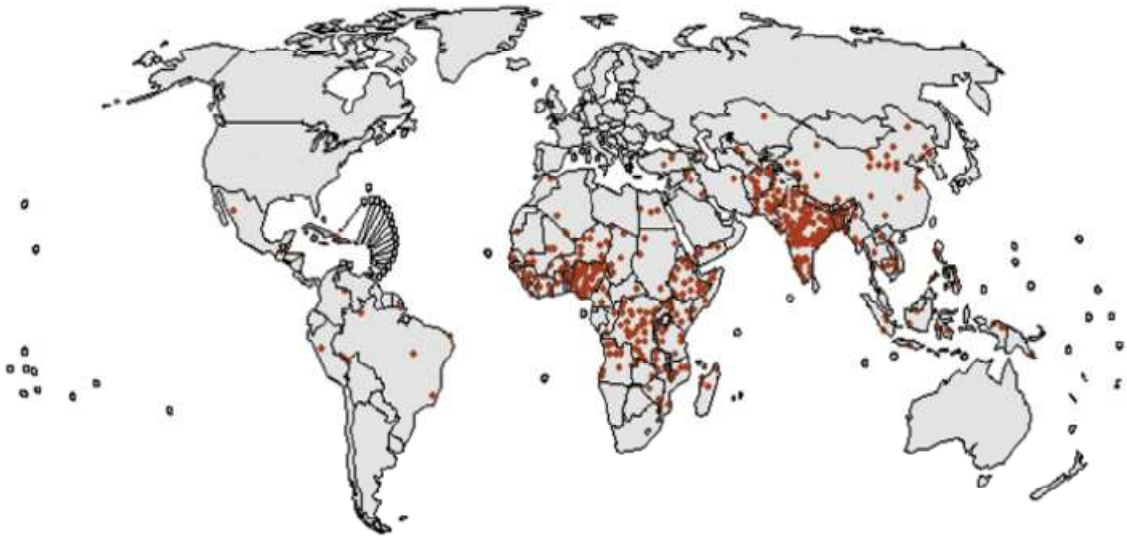

Figure 3: Global distribution of mortality associated with rotavirus disease, among children younger than 5 years of age, in 2004. Each dot represents 1000 deaths (90).

Rotavirus disease is also a major burden in Africa, where 140,000 – 150,000 children younger than five years die annually (54). A study regarding the burden of RV disease in Middle East and North Africa revealed that, in certain countries in the region, 112 cases per 100,000 were fatal. The hospitalization costs varied from $1.8 to $4.6 million annually, depending on the country (31).

1.8.1.2. Developed countries: an overview

Although rotavirus diarrhoea also occurs with high frequency in the developed countries, the mortality is low. In the USA, for example, mortality attributable to rotavirus is less than 40 paediatric deaths annually (5, 45). However, rotavirus causes serious morbidity within USA children under 5 years of age, being responsible for 3 million episodes of diarrhoea illness, 500,000 visits to the doctor and 60,000-70,000 hospitalizations every year (5). Overall, RVs cause 5-10% of all diarrhoeal episodes in infants and children aged less than 5 years, accounting for 30- 50% of severe diarrhoea cases (5, 84). The resulting financial burden associated with RV diarrhoea is estimated to be $200-$500 million on the USA health-care system, and $1 billion to the society (5, 32, 45).

In European Union (EU) countries, it was estimated the occurrence of 3.6 million episodes of rotavirus disease per year among the 23.6 million children younger than 5 years of age in the EU. In addition, every year in the EU, rotavirus accounts for 231 deaths, more than 87,000 hospitalizations and almost 700,000 outpatient visits (91). The estimated pre-vaccination burden of RV disease was particularly higher in Belgium compared to other European countries, even if age distribution was similar to neighbouring countries (92). In Belgium, rotavirus was estimated to account for about 26,800 outpatients visits and 5,600 hospitalizations annually in children younger than 7 years of age, which represents direct costs of 7.7 million euro and indirect costs of 12.8 million euro (93).

1.8.2. Age distribution

By the time they reach 3-5 years old, nearly all children will have experienced at least one RV infection, and while not every infection (including the first one) is

symptomatic, 1 in 5 cases will be presented to a doctor, 1 in 65 will require hospitalization and approximately 1 in 293 will be fatal (8, 30, 31, 84, 94, 95). The highest incidence of RV infection occurs between 6 and 24 months of age. Many of these infections result from household spread, others from the child care-related contacts and some through nosocomial exposure (45, 92, 94). Nosocomial rotavirus infection is very common, mostly because RVs are highly resistant to eradication by soap and alcohol-based gels. Although nosocomial RV infection affects more frequently infants between 3 months and 3 years of age, it can also occur in neonates, older children and the elderly (70, 83, 96). The low incidence of RV disease in infants aged less than 3 months may be related to the transplacental transfer of maternal antibodies or breast milk antibodies (55, 70, 97, 98). Neonatal RV infections are also believed to be asymptomatic, which may provide protection against severe rotavirus diarrhoea in later infancy (83, 99). The incidence of RV infection decreases progressively towards adulthood as immunity acquired in childhood protects most adults (95). However, rotavirus can also affect adults, which is often manifested in subclinical forms (94).

1.8.3. Seasonality

In countries with temperate climates, RV infections display a characteristic seasonal pattern, with epidemic peaks ranging from late autumn to early spring. In contrast, RV infections are rarely detected in summer (6, 23, 100). Although epidemiologists have attempted to associate disease incidence with climatological factors (rainfall, humidity and temperature), the variability of rotavirus epidemic patterns could not be explained by such factors (82, 100). Nevertheless, a more recent study, conducted by Pitzer and colleagues in the USA, showed that seasonality of rotavirus epidemics can be explained by spatiotemporal variation on birth rate. According to this study, epidemics tended to occur earlier (late November to early December) in localities having higher birth rates. In contrast, when the birth rate was low, epidemics occurred on average three months later (101).

In the USA, rotavirus diarrhoea shows characteristic winter-spring seasonality, spreading from the west to the east. Rotavirus disease occurs first in the southwest of

USA during the fall, and spreads progressively across the country, so that by late winter and spring, it has reached the northeast (37, 102, 103). Rotavirus epidemic season in European countries has a typically winter peak, with a gradual shift of rotavirus peak activity similar to the one observed in USA (south-western to the north and north-eastern direction). The peak of RV infections occurs first in Spain, usually in December, moving to France in February, and reaching Northern Europe, in particular, the Netherlands and Finland, in March (30, 104). According to a study conducted in Belgium during 1981-2002, rotavirus detection rate reached 54% in the first three months of the year (105).

The usual seasonal pattern of RV infection observed in the temperate regions (winter and spring peaks) does not occur uniformly in other areas of the world (5). In countries with tropical or subtropical climates, RV infections occur all year-round, with or without any major peak periods (6, 23, 106). The countries in the tropics almost always have high birth rates, which, one again, may help explain why rotavirus seasonality is less marked in such settings (101).

1.8.4. Genotype distribution

Rotavirus strains circulating in different geographical locations and time periods are characterised by a great genetic diversity (10, 22). The major human G-types are G1, G2, G3, G4, and G9, which, combined with the P-types P[8], P[4], and P[6], account for >80% of rotavirus-associated gastroenteritis episodes worldwide (1, 10, 62, 82). The distribution of these five globally important rotavirus genotypes can change drastically in a certain region from one year to another or during the same year in different geographical locations (6, 23). In fact, a particular genotype that is predominant in one season may be less active or even absent in the following season, and strains undetected in one year may appear in the next year (30). Despite this, G1P[8] strains still remain the most prevalent genotype in humans worldwide (10, 23).

Before 1995, G1P[8], G2P[4], G3P[8] and G4P[8] were the most important circulating genotypes in humans around the world (6, 23). Review analysis of studies published until 2005 revealed that these four major genotype combinations represented 90% of the rotavirus infections in Europe, North America and Australia,

compared with 68% in South America and Asia, and 50% in Africa. Moreover, G1P[8] strains single-handedly accounted for more than 70% of RV infections in North America, Europe and Australia, 30% of the infections in South America and Asia, and only 23% in Africa (107, 108).

G9 rotaviruses, previously isolated only sporadically, started to emerge after 1995. The G9 strains exhibit a high level of reassortment, and so, they can be found in a variety of combinations with P[8], P[6], P[11] and P[4] genotypes, although the most common is G9P[8] (6, 79). Over the years, G9 rotaviruses were able to spread rapidly across the entire globe, causing many national outbreaks in which they accounted for more than 50% of the RV gastroenteritis cases. As a result, G9 is now recognized as the fifth globally important rotavirus genotype (1, 6, 23, 79). In Europe, G9 rotaviruses have been found in several countries such as Belgium, Hungary, United Kingdom, Italy, Ireland, and The Netherlands (109-114). Moreover, in Portugal, G9P[8] was the predominant strain in a hospital-based study in 2006, accounting for 90% of the cases (115). In non-European countries, G9 rotaviruses have been identified, for instance, in Thailand, Brazil, Argentina, India, Bangladesh and Japan (116-121).

More recently, G12 rotaviruses, which are normally associated with either P[8] or P[6], have been identified in sporadic cases and outbreaks in countries around the world, for example, in Bangladesh, India, South Africa, Argentina and Hungary (82, 122-125). The possible global spread and increasing epidemiologic importance of these RVs suggests that G12 might be the next emerging genotype among humans (1, 6, 25).

In these past years, several epidemiological studies have reported the existence of some uncommon rotavirus types (108). Examples include G5P[8], G8P[4], G8P[8] and G10P[9] strains in Brazil; G3P[6] and G4P[6] strains in Mexico and South Africa; G8P[6] in Malawi, and G2P[6] strains in Guinea-Bissau (126-133). It is important to note that the G5P[8] strains in Brazil and the G8P[6] strains in Malawi seem to be regionally prevalent (25).

1.9. Prevention and control

1.9.1. Control

Although faecal-oral transmission seems to be the primary means of acquisition and transmission of rotavirus infection, fomites and virus-contaminated hands also seem to be implicated in the spread of the virus, especially because rotavirus can remain viable on inanimate surfaces for many days and survive for several hours on human hands (45, 70). Handwashing with soap and water has proven to be relatively ineffective in reducing the amount of viruses on hands (45, 70). Rotaviruses are also able to withstand many commercially-available disinfectants and antiseptics, including quaternary ammonium-based products frequently used in medical institutions (70, 134). Chlorhexidine, for example, demonstrates low activity against RVs. Moreover, even though studies have shown that alcohol-based hand gels may be more effective than soap, none of them seem to eradicate completely the organism from human hands (70). In contrast, 95% ethanol is perhaps one of the most effective disinfectants that can inactivate rotavirus (134). Likewise, a commercially available high alcohol-containing disinfectant spray (0.1% o-phenylphenol and 79% ethanol) has proven to be very effective for use on fomites (45, 70).

1.9.2. Vaccination

Vaccination is the most efficient method for preventing RV infection (94). The main goal of rotavirus vaccination programmes is to prevent death and severe disease caused by rotaviruses. This is extremely important in developing countries, where the burden of disease is heavier and the mortality rate is highest. In addition, vaccine programmes also seek to reduce the large number of emergency consultations and hospitalizations and, consequently, reduce the costs (direct and indirect) associated with acute rotavirus disease (44, 97).

Clinical research to develop a safe, effective rotavirus vaccine began in the mid-1970s and, since then, several vaccine candidates were investigated (97). The first commercial rotavirus vaccine was RotaShield® (Wyeth Lederle), which was a live, orally administrated, tetravalent rhesus–human reassortant vaccine containing a mixture of four virus strains representing the most common G types, G1 to G4. Extensive trials

indicated that this vaccine was safe and efficacious. Consequently, RotaShield® was licensed in the USA in 1998 and recommended for routine use in US infants shortly afterwards. However, in less than a year the vaccine was withdrawn from the market due to an excess number of reports of intussusceptions in infants following vaccination (44, 55, 97, 135, 136). Intussusception is a serious medical condition in which a part of the intestine invaginates upon itself, resulting in obstruction, followed by local necrosis of gut tissue (55).

Currently, two new live, oral vaccines have been licensed for the prevention of rotavirus-associated gastroenteritis: a monovalent human rotavirus vaccine (Rotarix® from GlaxoSmithKline) and a pentavalent human-bovine reassortant vaccine (RotaTeq® from Merck). Both vaccines have demonstrated to be safe and highly effective in large-scale clinical trials conducted in several countries. Thus, the World Health Organization (WHO) now strongly recommends the inclusion of rotavirus vaccination into the national immunization programmes of countries where these vaccines have shown a significant public health impact and where appropriate infrastructure and financing mechanisms are available to sustain vaccine utilization (44, 55, 82).

1.9.2.1. Rotarix ®

The monovalent human rotavirus vaccine, Rotarix®, is based on a live attenuated human G1P[8] rotavirus strain, which belongs completely to the Wa-like genogroup, and represents the most common human rotavirus VP7 and VP4 genotypes (23, 44). A live-attenuated vaccine was originally developed in Cincinnati, Ohio, by passage in tissue culture of a wild-type human rotavirus (strain 89-12) isolated from a naturally infected child with rotavirus gastroenteritis. This vaccine was further developed by GlaxoSmithKline, who modified the parent strain by cloning and multiple passages in tissue culture, resulting in the attenuated Rotarix® vaccine strain (RIX4414) (55, 97). The vaccine is administered orally in a two-dose schedule, with the first dose administered at 6-12 weeks of age, and the second dose administered following an interval of at least 4 weeks (44).

Rotarix® was tested for safety and efficacy in a phase III clinical trial involving 63,225 infants from Finland and eleven Latin American countries. The vaccine was found to be safe, immunogenic and highly efficacious in protecting infants against severe rotavirus gastroenteritis, and there was no association with an increased risk of intussusception. Rotarix® also demonstrated a protection rate of 85% against severe RV diarrhoea and against rotavirus-associated hospitalization, reaching the 100% against the most severe dehydrating RV gastroenteritis episodes. Furthermore, this vaccine showed an efficacy of 92% against G1P[8] strains, and 87% against strains sharing only the P[8] antigen (G3P[8], G4P[8] and G9P[8]). In addition, the efficacy of the vaccine against G2P[4] strains was found to be 41%, but this result was not significant in this trial. However, in a subsequent meta-analysis of more clinical trial data (combined results from phase II and phase III studies), the overall efficacy of Rotarix® against the G2P[4] type was 67%, indicating that the vaccine can also protect, although to a lesser extent, against strains that do not share its VP4 or VP7 proteins (137).

In a randomized, double-blind, placebo-controlled study conducted in six European countries, Rotarix® was found to be highly immunogenic. Through one rotavirus season, efficacy of Rotarix® against RV gastroenteritis of any severity was 87% and against severe RV gastroenteritis was 96%. The combined efficacy of the vaccine for two whole seasons was 90% against severe disease. For any rotavirus gastroenteritis, through two rotavirus seasons, Rotarix® had efficacies of 90%, 58%, 85%, 83% and 73% against G1, G2, G3, G4, and G9, respectively (138).

Rotarix® was first licensed in Mexico and the Dominican Republic in 2004 and is now approved in at least 90 countries worldwide, including European Union and most of Latin America countries (2006), USA (2008), African and Asian countries (55, 92, 135).

1.9.2.2. RotaTeq®

Rotaviruses ability to reassort during co-infection *in vitro* has been exploited to develop attenuated reassortant vaccines with specific combinations of gene segments derived from each of the parental rotavirus strains - an animal strain and a human strain. This modified Jennerian approach has been used to develop the pentavalent

rotavirus vaccine (12). RotaTeq® contains five human-bovine reassortant rotavirus (G1, G2, G3, G4, and P[8]) on the backbone of the naturally attenuated tissue culture-adapted parental bovine rotavirus (BRV) WC3 strain (G6P[5]). Four reassortant rotavirus express one of the VP7 proteins G1, G2, G3 or G4 from the HRV parent strains and the VP4 protein P[5] from the BRV strain. The fifth reassortant virus expresses the VP7 protein G6 from the BRV parent strain and the VP4 protein P[8] from the HRV strains (12, 44, 55, 97).

RotaTeq® is routinely recommended as a three-dose schedule, with the first dose administered at 6-12 weeks of age and subsequent doses administered at 4–10 week intervals (44, 97). The efficacy and safety of RotaTeq® have been demonstrated in two phase III trials, including a large clinical trial of more than 70,000 infants enrolled primarily in the USA and Finland. The study revealed that RotaTeq® was well tolerated, and no vaccine-associated serious adverse events were reported, including intussusception. In addition, RotaTeq® reduced the incidence of medical office visits by 86%, emergency department visits by 94% and the incidence of rotavirus gastroenteritis hospitalizations by 96%. Through the first rotavirus season after vaccination, the efficacy of the vaccine against G1-G4 RV gastroenteritis of any severity was 74% and against severe G1-G4 RV gastroenteritis was 98%. In the second rotavirus season post-vaccination, the efficacy of RotaTeq® was 63% against any RV gastroenteritis and 88% against severe disease. Efficacy against disease caused by G1 strains was 75% and against disease caused by G2 strains was 63%. The results for the other genotypes (G3, G4 and G9) were not statistically significant due to the small number of patients in each category (139).

RotaTeq® was licensed in USA by the Food and Drug Administration (FDA) in 2006, and is now approved in more than 100 countries around the world (97). In 2010, the data from RotaTeq® efficacy trials in sub-Saharan Africa and Asia finally became available. The overall efficacy of the vaccine against severe RV gastroenteritis in Asian and African countries was 48.3% and 39.3%, respectively. Although the efficacy noted in these trials were significant, both estimates of efficacy were inferior to those reported in trials of RotaTeq® in more industrialized countries. This lower effectiveness in developing settings might be explained by differences in study design, collection procedures, and clinical scoring systems (140, 141).

1.9.3. Rotavirus Surveillance in Europe

Surveillance is important to determine the burden of rotavirus disease and the characteristics of the strains circulating in different countries. As RV vaccination continues to increase worldwide, global surveillance of rotavirus has become critical to monitor the impact of RV vaccines on morbidity and mortality, describe genotype distributions in different countries, and identify emerging RV strains that might escape vaccination. Moreover, surveillance is also important to evaluate whether strain variability is a natural occurrence or whether it is the result of a potential selection of RV genotypes through vaccine pressures (142, 143). Hence, the development of surveillance networks such as the EuroRotaNet, which now covers many central and eastern European countries (95).

The European Rotavirus Network (EuroRotaNet) was established in January 2007 and consists in a network of European laboratories collaborating in a study which will gather comprehensive information on the rotavirus genotypes co-circulating throughout Europe. Currently, the EuroRotaNet database contains data from the 17 European countries that are part of the network (Denmark, Finland, France, Germany, Hungary, Italy, The Netherlands, Slovenia, Spain, Sweden, United Kingdom, Belgium, Bulgaria, Lithuania, Greece, Romania and Austria), and data from some countries who are not formal members of the network. Each record uploaded into the database comprises epidemiological data (age, sex, geographical location, date of sample collection, and clinical symptoms) linked to the genotyping data. Genotyping is performed by a designated laboratory in each country. Furthermore, a web-accessible database (http://www.eurorota.net/) was developed in order to share network information between participants. Since rotavirus vaccination in Europe is not being introduced equally, EuroRotaNet will permit comparisons of genotype distributions between neighbouring countries with and without rotavirus vaccination programmes (144).

1.10. Aims and objectives

Until 2006, the percentage of RV positive cases monitored at Gasthuisberg University Hospital (GUH) was relatively stable. However, due to the introduction of Rotarix® (2006) and RotaTeq® (2007) into the Belgium market, and the consequently growing number of vaccinated children, this percentage dropped drastically in the following rotavirus seasons post-vaccination. Up until now Rotarix® remains the vaccine with the higher uptake in Belgium. In contrast, since Rotarix® introduction, the following rotavirus seasons (2006-2007, 2007-2008 and 2008-2009) have been marked by a relative increase on the prevalence of G2P[4] rotavirus strains (23). Hence, we hypothesized that this event may be vaccine-related, since Rotarix® is the most used vaccine in Belgium, and this vaccine offers better protection against strains associated with P[8] than against strains associated with P[4]. The aim of this project was to do an epidemiological study on rotavirus prevalence and genotype distribution in Belgium in the 2009-2010 season, as well as to verify if this further relative increase in G2P[4] rotavirus strains could be due to vaccine selective pressure, or if it was attributable to normal genotype fluctuations.

2. MATERIALS AND METHODS

Between August of 2009 and July of 2010, 577 stool samples were collected from paediatric patients from all over Belgium. In this study, we report the analysis and characterization of these samples, which comprises a series of consecutive procedures, each one described below.

2.1. Samples dilution

In order to stabilize the faecal samples, approximately 200 mg of faeces were diluted in 750 mL of Viral Transport Medium (VTM), in 1.5 mL tubes. For less than 200 mg, the faeces were diluted in 500 mL of VTM. Afterwards, the content of the tubes was mixed by pulse-vortexing and stored at -80°C.

2.2. Viral RNA extraction

Viral RNA was extracted from the diluted samples using the QIAamp Viral RNA Mini Kit (Qiagen/Westburg, The Netherlands), according to the manufacturer's instructions. The tubes containing the RNA extract were stored at -20°C.

2.3. Reverse Transcription - Polymerase Chain Reaction

The RT-PR procedure was carried out using the Qiagen OneStep RT-PCR Kit (Qiagen/Westburg, The Netherlands). The primer pairs used were BEG9 (forward primer) and END9 (reverse primer) for VP7, and VP4_1-17F (forward primer) and CON2 (reverse primer) for VP4 (Eurogentec, Belgium). The primers sequences are available in Table 2.

Table 2: Sequences of the primers for VP4 and VP7 (79).

Gene	Primer	Sequence
VP4	VP4_1-17F	5'- GGC TAT AAA ATG GCT TCG C - 3'
VP4	CON2	5'- ATT TCG GAC CAT TTA TAA CC - 3'
VP7	BEG9	5'- GGC TTT AAA AGA GAG AAT TTC CGT CTG G - 3'
VP7	END9	5'- GGT CAC ATC ATA CAA TTC TAA TCT AAG - 3'

Initially, the RNA extracts, primers, RT-PCR buffer and dNTP Mix were thawed at room temperature. After thawing, 6 µL of RNA extract were diluted in 6 µL of MQ-water. Furthermore, a working solution for each primer (VP4_1-17F and CON2 for VP4; BEG9 and END9 for VP7) was also prepared (10 µM; 1:6 dilution of stock solution). The RT-PCR mix, whose formula is described below in Table 3, was prepared in 0.2 mL tubes placed beforehand in a PCR-rack.

Table 3: Master mix for RT-PCR.

Reagents	Volume (µL) per reaction
MQ-water	22
QIAGEN OneStep RT-PCR **Buffer**(5x)	10
QIAGEN **dNTP Mix** (10 mM of each dNTP)	1
Fw Primer (10 µM)	3
Rv Primer (10 µM)	3
QIAGEN OneStep RT-PCR **Enzyme Mix**	1

Afterwards, the RNA templates were denatured at 95°C for 2 min and transferred immediately to ice. Then, 10 µL of each RNA template were added to the corresponding RT-PCR tube containing the 40 µL of master mix. The tubes were kept on ice until they were loaded on the Thermocycler Biometra T3000 (Biometra, The Netherlands), where a program was set for each gene segment (VP4 or VP7) with the parameters shown in Table 4. Once the RT-PCR process was complete, the RT-PCR products were kept at 4°C or stored at -20°C.

Table 4: RT-PCR parameters for VP4 and VP7.

Step	Time period	Temperature
Reverse transcription	30 min	50°C
Initial PCR activation step	15 min	95°C
3-step cycling		
Denaturation	30 sec	94°C
Annealing	30 sec	45°C (VP4) or 50°C (VP7)
Extension	90 sec	72°C
Number of cycles: 35		
Final extension	10 min	72°C
Cooling	-	4°C

2.4. Polyacrylamide Gel Electrophoresis (PAGE)

2.4.1. Preparation of the gels

The polyacrylamide gel solution was prepared as follows: first, 22.5 mL of 29:1 AA/BA (Biorad, Belgium) were mixed with 15 mL of 10x TBE buffer (Invitrogen, UK) and 6 mL of 1.6% (v/v) APS (Merck) in a graduated cylinder; then, MQ-water was added until 150 mL; finally, 150 µL of TEMED (Life Technologies, Belgium) was mixed with the solution. Afterwards, the solution was poured into a mould to make 10 sheets of gel, each one with 15 wells. Once the cross-polymerization was completed, the gels were stored at 4°C.

2.4.2. PAGE

The PAGE gel was placed into an electrophoresis apparatus SE 250 Mighty Small II Unit (Pharmacia, Sweden) and the reservoir of the device was filled with 1x TBE buffer (Invitrogen, UK). First, 3 µL of Molecular Weight Marker XIV (Roche Applied Science, Belgium) were loaded in one well of the gel. The marker was previously prepared by mixing 50 µl of 10x TBE buffer and 50 µl of loading dye (90% (v/v) formamide; 50 mM Tris-borate pH 7.5; 1 mM EDTA; 0.1% (v/v) xyleencyanol (light blue); 0.1% (v/v) bromophenol blue (dark blue)) in 200 µl of MQ-water. For each sample, 9 µL of RT-PCR product were mixed with 1 µL of loading dye in a small well plate, and the samples were then loaded into each well of the gel. After ensuring that all wells were covered with TBE buffer, the PAGE was performed at 200 mA and 200 V for 36 min using the Power Source 300V Power Supply (VWR, Belgium).

2.4.3. Staining of the nucleic acids

To stain the nucleic acids, the gel was immersed on ethidium bromide (EtBr) for a few minutes: 50 µL of EtBr 10 mg/ml (Invitrogen, UK) in 200 ml TBE-buffer. Afterwards, the gel was taken out of the ethidium bromide and irradiated with UV light on the Red Personal Gel Imaging System (Westburg, Belgium). Samples with bright bands and the correct molecular weight for the target gene were considered positive for rotavirus, and as a result, purified and sequenced.

2.5. Purification

In order to remove unincorporated primers, dNTP's, buffers and enzymes, the RT-PCR products positive for rotavirus were purified with the MSB® Spin PCRapace Kit (Invitek, Germany) or InnuPREP PCR pure kit (Analytik Jena, Germany), according to manufacturer's instructions. The tubes were stored at -20°C.

2.6. Sequencing

For the sequencing reaction, a mix was prepared in 0.2 mL tubes, as described below in Table 5. The tubes were then loaded in the Biometra T3000 Thermocycler (Biometra, The Netherlands) with the following sequencing program: 25 cycles of 30 sec at 96°C, 15 sec at 50°C and 4 minutes at 60°C.

Table 5: Composition of the sequencing mix.

Reagents	Volume (µL) per reaction
BigDye™ (Applied Biosystems, USA) Contains AmpliTaq™ DNA polymerase, dNTPs and fluorescently labeled chain-terminating ddNTPs)	2
BigDye Terminator 5x Sequencing Buffer (Applied Biosystems, USA)	2
Forward primer 10 µM (VP4_1-17F for VP4 or BEG9 for VP7) 1:10 dilution of stock solution	1
RT-PCR purified product	5

After the sequencing reaction, an ethanol/NaAc precipitation was performed on the sequencing products to remove dye terminators, primers, buffers and enzymes. First, a mix consisting of 62.5 µL of 100% (v/v) ethanol (VWR, Belgium), 24.5 µL of MQ-water and 3 µL of NaAc (3 M; pH 4.6), was prepared per reaction. After that, 90 µL of this solution and 10 µL of sequencing product were added to 1.5 mL tubes and mixed by pulse-vortexing. The tubes were centrifuged at 13,000 rpm for 30 min. Afterwards, a pellet of precipitated DNA was formed in the bottom of the tubes and the supernatant was carefully aspirated and discarded. To wash the pellets, 150 µL of 70% (v/v) ethanol were added to the tubes, which were then centrifuged at 13,000 rpm for

5 min. After centrifugation, the supernatant was once again removed and the remaining drops were left to dry at 50°C. Finally, 16 µL of formamide were added to the dry pellets, followed by heat denaturation at 95°C for 2 min. Afterwards, the tubes were stored immediately at -20°C and kept at that temperature until the samples were loaded in the ABI PRISM^{TM} 3100 automated sequencer (Applied Biosystems, USA).

2.7. Sequence and data analysis

The chromatogram sequence files for VP4 and for VP7 were analysed with Chromas 2.3 (Technelysium, Australia), corrected manually and compared with sequences available in GenBank by performing a BLAST analysis. Data analysis and its display were carried out using Microsoft Office Excel 2007 (Microsoft, USA), MEGA 4.0 (Center for Evolutionary Functional Genomics, USA) and MapInfo Professional 9.0 (Pitney Bowes, USA).

3. RESULTS

A total of 577 stool samples were collected from paediatric patients from all over Belgium, between August of 2009 and July of 2010. Rotavirus antigens, i.e. PCR positives for VP4 and VP7 (see Appendix A), were identified in 491 samples, which corresponded to a detection rate of 85%.

3.1. Distribution of P and G genotypes

Genotyping was performed by BLAST searches on GenBank database with the VP7 and VP4 gene sequences, for G-typing and P-typing, respectively (see Appendix B). Out of the 491 positive samples, four were untypeable for the VP7 protein, which corresponds to a percentage of 0.8%. Overall, G2 (61.9%) was the most prevalent genotype (Figure 4), followed by G1 (25.3%), G4 (3.7%), G9 (3.3%) and G12 (2.9%). Minor genotypes were also detected: nine G3 strains (1.8%) and two G6 strains (0.4%).

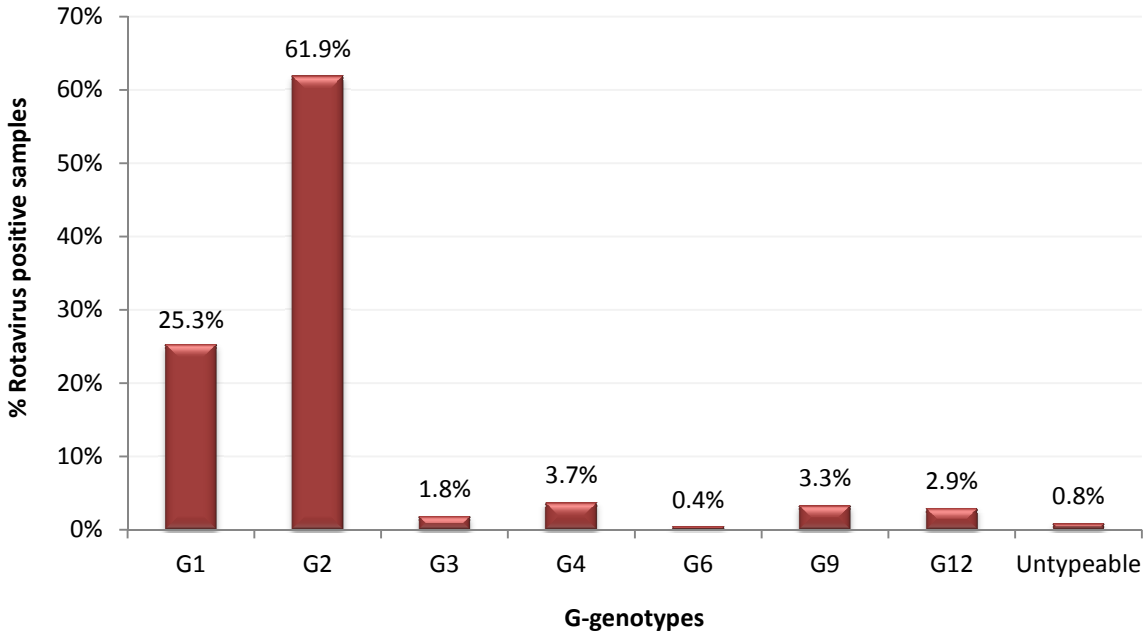

Figure 4: G-genotype distribution in Belgium in the 2009-2010 season.

P-genotyping was performed for all samples as well, from which seventeen samples were untypeable for VP4, which corresponds to a percentage of 3.5%. The

most prevalent genotypes were P[4] (60.9%) and P[8] (34.6%). A few unusual P-types (three P[6] strains and two P[14] strains) were also detected (Figure 5).

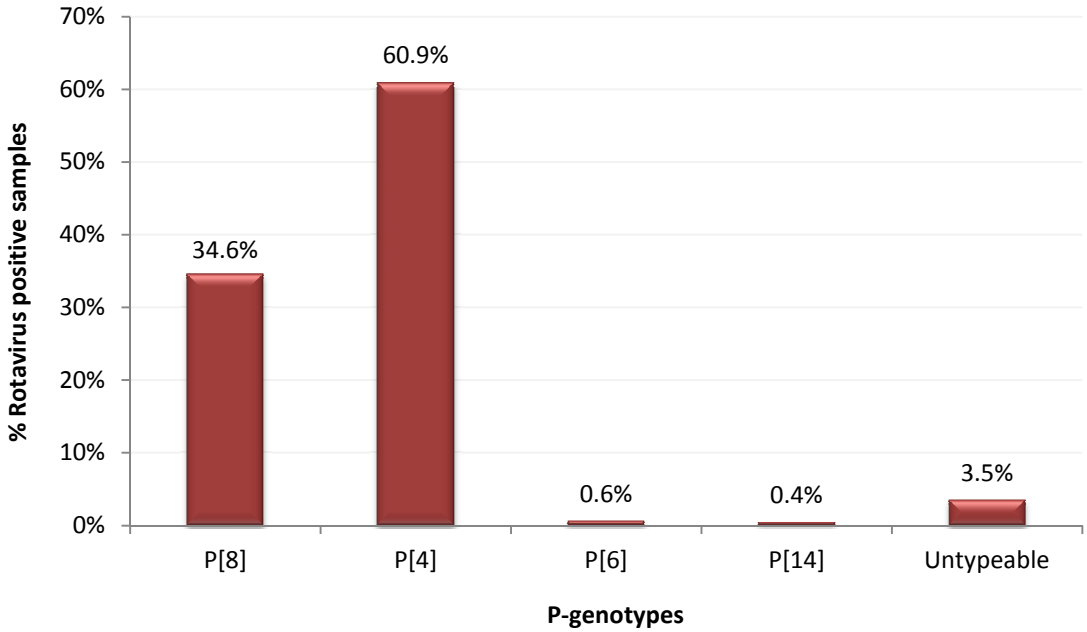

Figure 5: P-genotype distribution in Belgium in the 2009-2010 season.

In general, G1, G3, G9 and G12 were mainly associated with the P[8] genotype, whereas all G2 and G4 were associated exclusively with P[4] and P[8], respectively. Uncommon genotype combinations found in the 2009-2010 season (Figure 6) include G1P[4] (four strains), G6P[14] (two strains), G9P[4], G3P[6], G12P[6], and G9P[6] (one strain each).

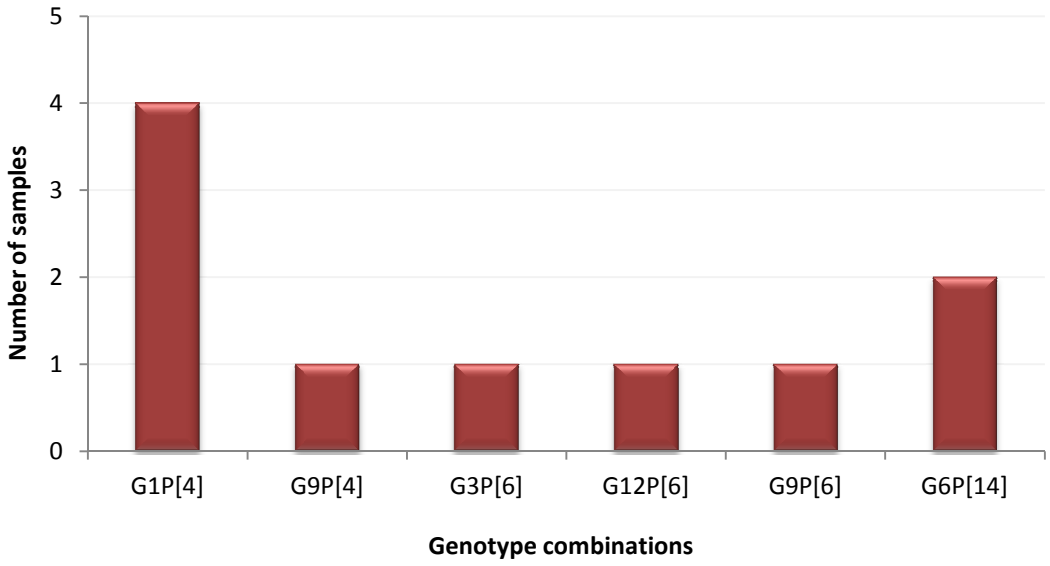

Figure 6: Uncommon genotype combinations identified in Belgium in the 2009-2010 season.

3.2. Geographical distribution of G genotypes

The geographical distribution of G-types in Belgium in the 2009-2010 season is shown in Figure 7. In total, thirty-four out of 491 samples (thirty samples without postcode and four samples G-untypeable) were excluded for this map analysis.

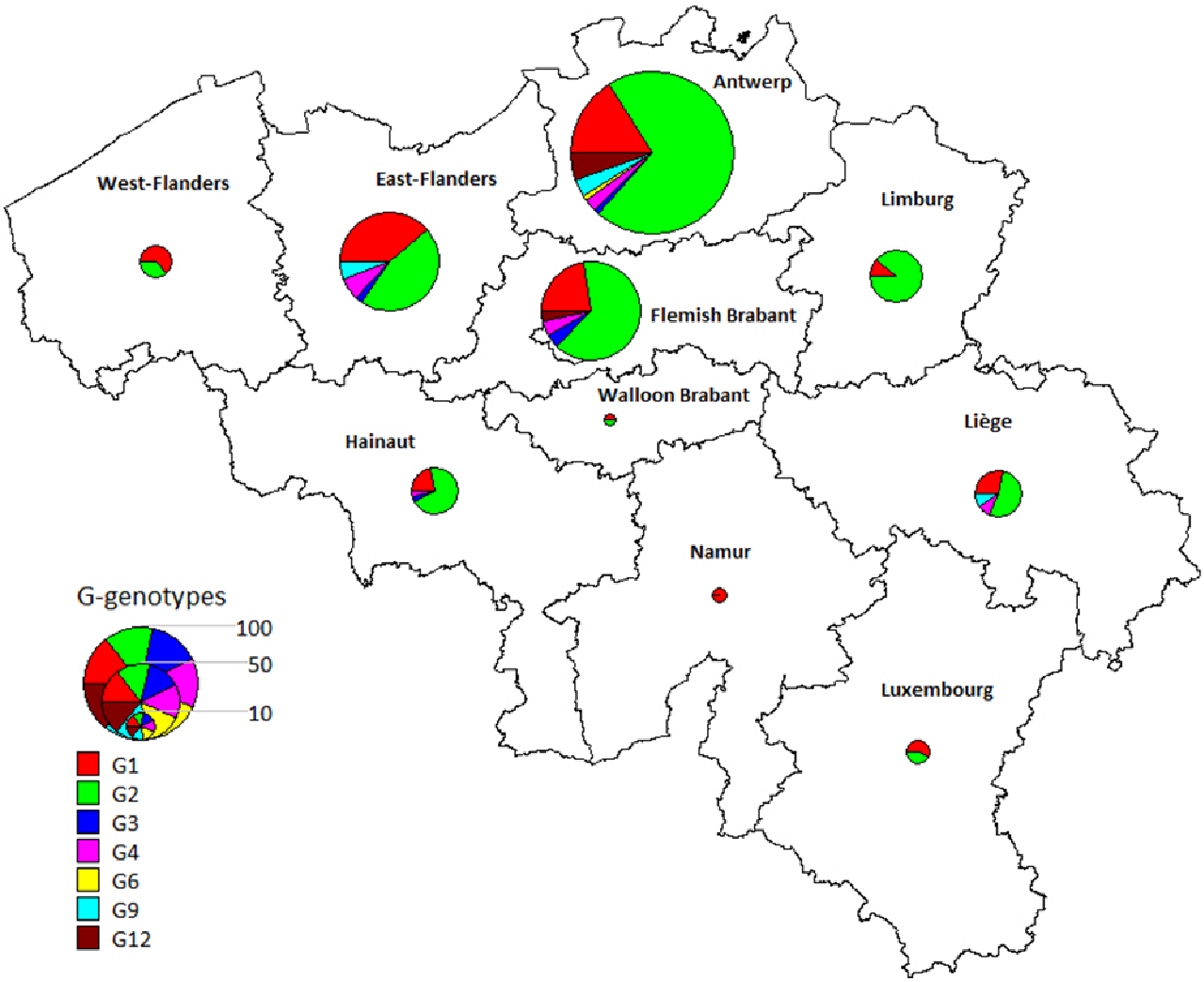

Figure 7: Geographical distribution of G-genotypes in Belgium in the 2009-2010 season. Unit: number of samples.

The majority of the rotavirus positive samples came from the north of Belgium, specially from the provinces of Antwerp (43.3%), Flemish Brabant (18.2%) and East-Flanders (17.9%). In nearly all provinces, G2 (represented in green on the pie chart) was the most prevalent genotype, whereas G1 (represented in red) was the second most prevalent. G1 genotype had a prevalence of 39% in East-Flanders (32 samples out

of 82), 28.6% in Liège (6/21), 22.9% in Flemish Brabant (19/83), 21.7% in Hainut (5/23), 16.2% in Antwerp (32/198), and 11.1% in Limburg (3/27). In contrast, G2 accounted for 88.9% of the samples in Limburg (24/27), 70.2% in Antwerp (139/198), 69.6% in Hainut (16/23), 63.9% in Flemish Brabant (53/83), 52.4% in Liège (11/21), and 45.1% in East-Flanders (37/82). Although G2 was the most predominant genotype, some regional variations can be seen on the map. For instance, in the provinces of West-Flanders and Luxembourg, G1 showed a higher prevalence than G2, whereas in Waloon Brabant G1 and G2 had the same prevalence. Moreover, in Namur all samples contained G1 specificity. However, due to the small number of samples ($n \leq 11$) from these provinces, these results cannot be considered significant.

As for the other genotypes, G3 (represented in blue) was found in Flemish Brabant (4 samples), Antwerp, East-Flanders (2 samples in each province), and Hainut (1 sample); G4 (represented in pink) was identified in samples from East-Flanders (6 samples), Antwerp (5 samples), Flemish Brabant (4 samples), Liège (2 samples) and Hainut (1 sample); G6 (represented in yellow) was only found in Antwerp; G9 (represented in cyan) was found in samples from Antwerp (7 samples), East-Flanders (5 samples) and Liège (2 samples); finally, G12 (represented in brown) was only found in Antwerp (11 samples) and Flemish Brabant (3 samples).

3.3. Evolution of genotype distribution in Belgium in the last three seasons

The evolution of genotype distribution in Belgium in the past three seasons (2007-2010) is shown in Figure 8. G2 was the most predominant genotype in the last three seasons, with a prevalence of 40.1% in 2007-2008, 47.8% in 2008-2009 and 61.9% in 2009-2010. G1 was the second most prevalent genotype in these seasons, accounting for 31.7% of the samples in 2007-2008, 13.8% in 2008-2009, and 25.3% in 2009-2010. In the 2007-2008 season, G9 was the third most prevalent genotype (13.2%), followed by G4 (8.7%) and G3 (3.6%), whereas in 2008-2009, G3 (13.4%) achieved a higher prevalence than G9 (11.3%) and G4 (8.5%). In 2009-2010, however, G3, G4 and G9 had relatively low frequencies, as mentioned previously. G12 genotype was found in 2007-2008 (1.4%) and 2009-2010 (2.9%), but not in 2008-2009.

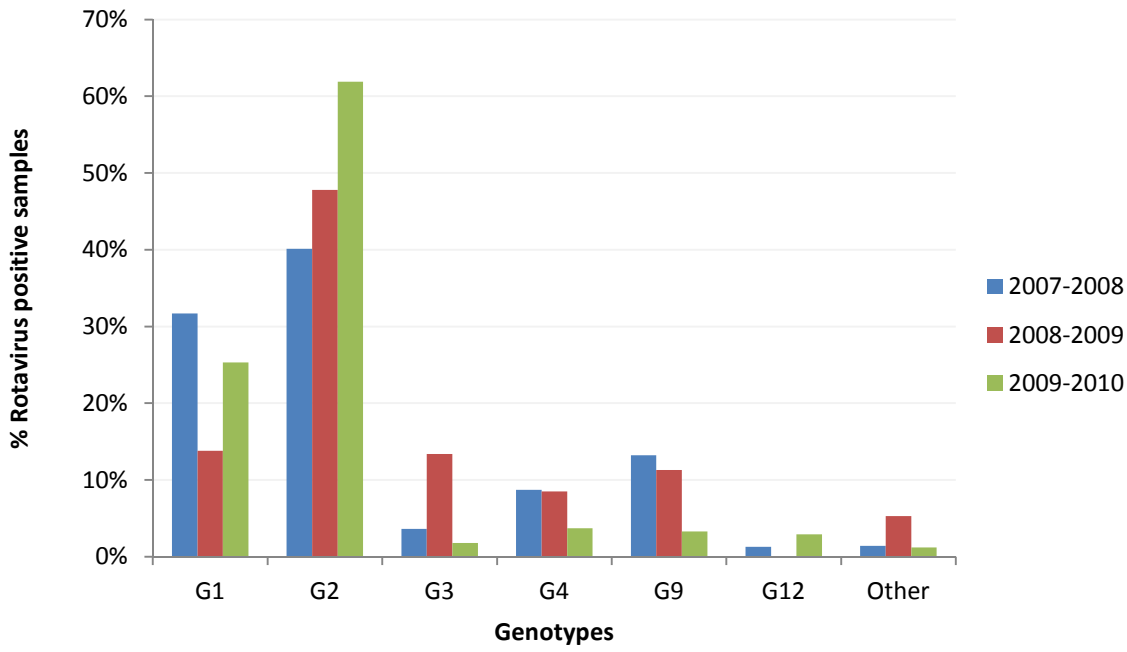

Figure 8: Distribution of the most common genotypes in Belgium in the seasons 2007-2008 (blue bars), 2008-2009 (red bars), and 2009-2010 (green bars).

3.4. Phylogenetic analysis of G2P[4] strains

Due to the further relative increase in the number of G2P[4], a phylogenetic analysis was performed on the nucleotide sequences of G2 and P[4] strains in order to identify potential different lineages co-circulating in Belgium. For the construction of the dendrograms, six short sequences were excluded in each analysis (see Appendix C for sequence alignment and Appendix D for the full phyogenetic trees).

The phylogenetic tree for G2 rotavirus strains (Figure 9) showed three major lineages: G2-I (138 samples), G2-III (118 samples) and G2-IV (37 samples). Two minor lineages were also identified: G2-II composed by four samples, and G2-V, which comprises merely one sample clustering differently from all others. Regardless, all G2 strains included in lineages I-IV seem more closely related to each other than to the G2 strain present in RotaTeq®.

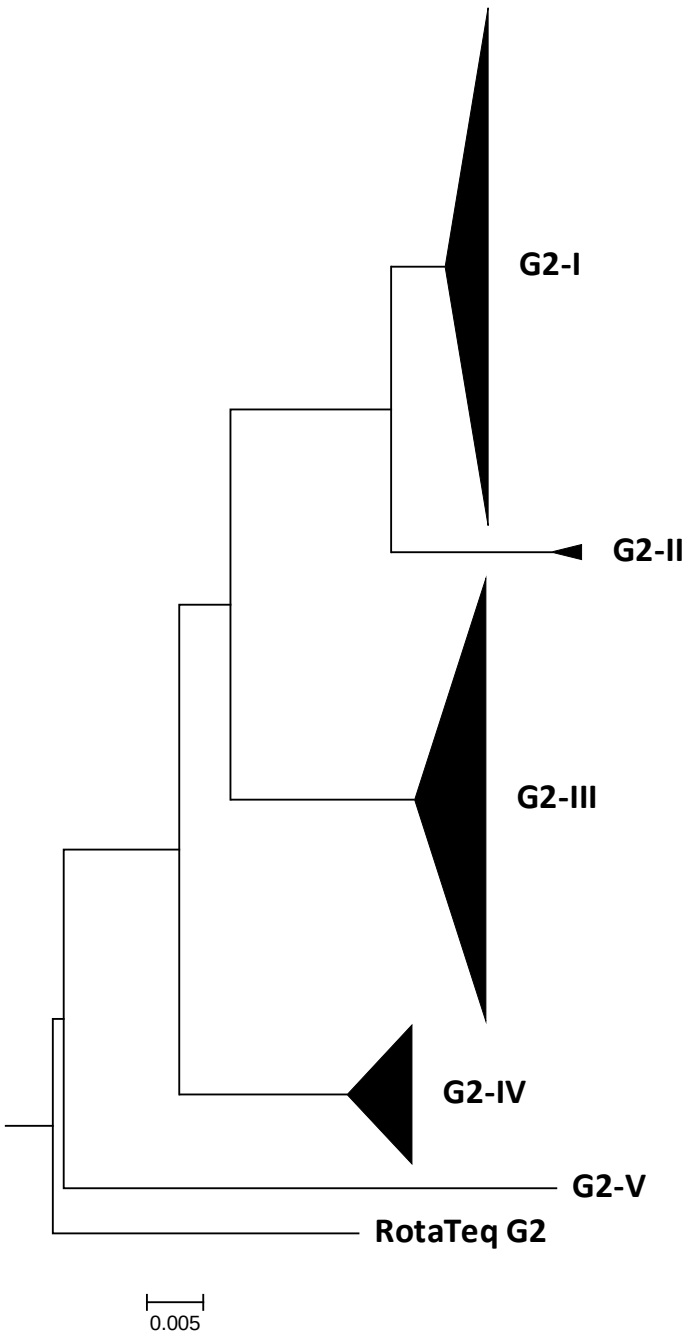

Figure 9: Neighbor-joining phylogenetic tree based on nucleotide sequences of the VP7 sequences for Belgian G2 strains in the 2009-2010 season. Distance estimation method used: Kimura 2-parameter model.

The phylogenetic tree for P[4] rotavirus strains (Figure 10) showed a similar number of clusters: two major lineages, P[4]-I (179 samples) and P[4]-III (105 samples), and four minor lineages, P[4]-II (4 samples), P[4]-IV (3 samples), P[4]-V (1 sample) and P[4]-VI (1 sample).

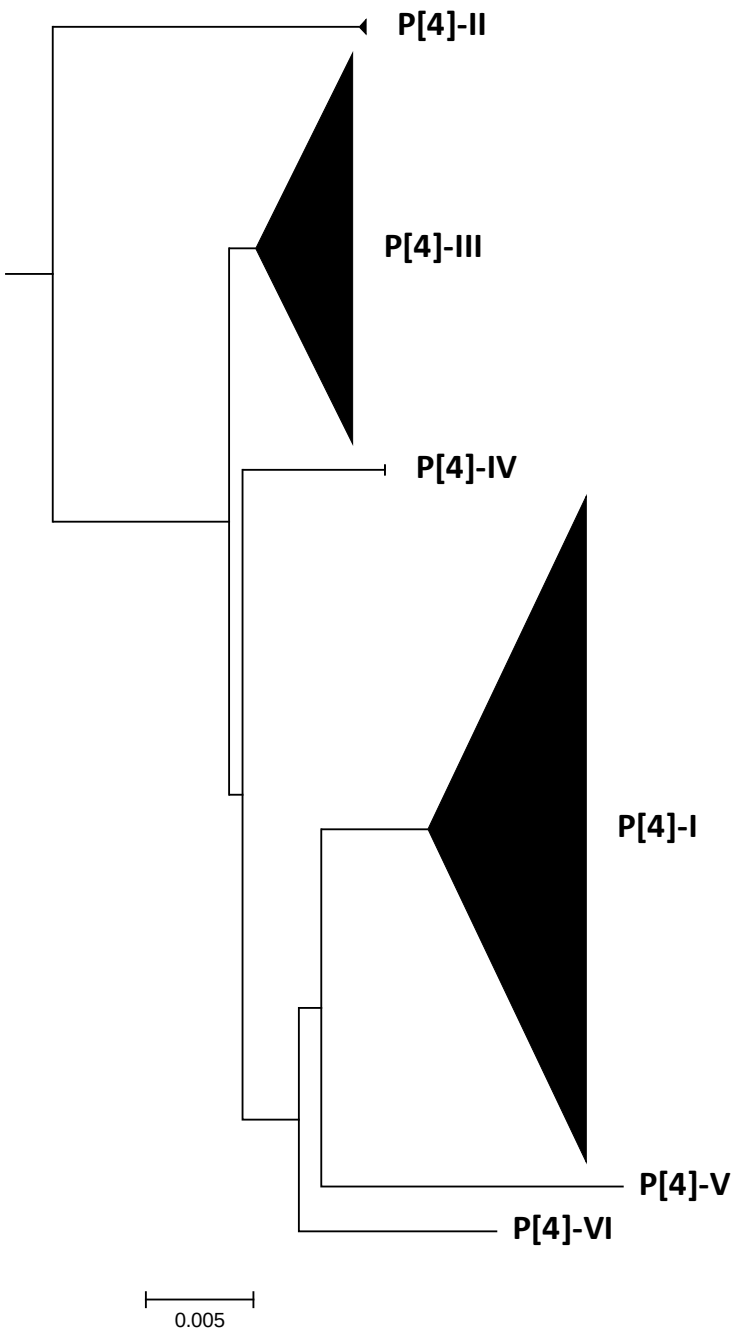

Figure 10: Neighbor-joining phylogenetic tree based on nucleotide sequences of the VP7 sequences for Belgian P[4] strains in the 2009-2010 season. Distance estimation method used: Kimura 2-parameter model.

After comparison of the different lineages obtained for G2 and P[4] strains, we confirmed that P[4]-I includes practically all samples from G2-I and G2-IV. Furthermore, lineages II and V were identical for both G2 and P[4], whereas lineage III was nearly identical. P[4]-IV was a small cluster including one sample from G2-I and

two samples from G2-III. The lineage P[4]-VI resulted from a P[4] associated with a G9 strain, which in turn made it cluster apart from the other P[4] strains. These differences suggest the presence of reassortment among the G2P[4] lineages.

3.5. Geographical distribution of G2 lineages

A geographical analysis was performed for the different lineages of G2 (G2-I, G2-II, G2-III, G2-IV and G2-V) to determine potential discrepancies in their location. Once again, only samples with postcode information could be used for this analysis.

G2-I was essentially found on the north of Belgium, mainly in the province of Antwerp, although some samples were also from the south of the country, namely from Hainaut, Liège and Luxembourg. In contrast, G2-II can only be found in the east, in the province of Liège (Figure 11).

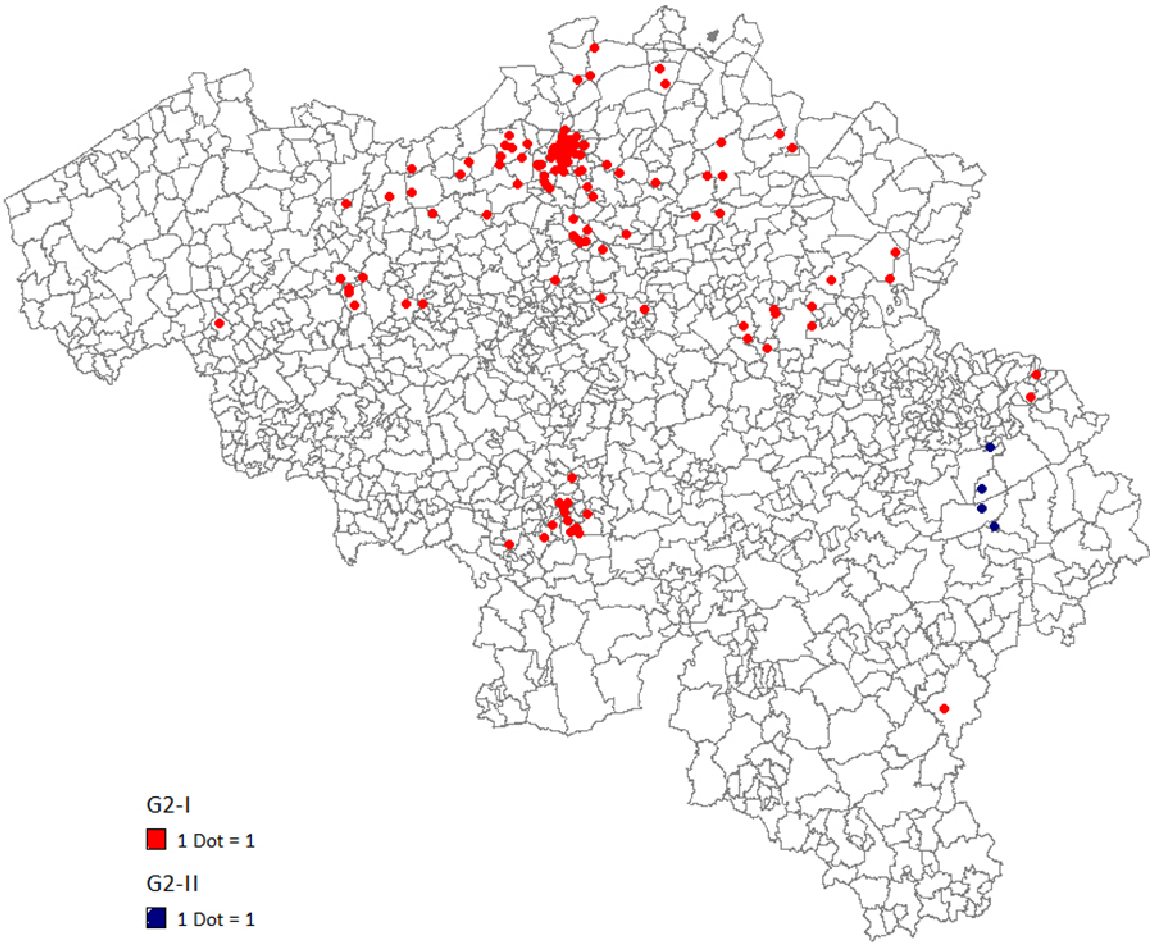

Figure 11: Geographical distribution of G2-I (red dots) G2- II (blue dots). Unit: number of samples.

The geographical distribution of G2 lineage III is represented in Figure 12. Just like the first cluster, G2-III is also mostly found in the north of Belgium, especially in the provinces of Antwerp, Flemish Brabant and Limburg. Additionally, G2-III includes some samples from the south of Belgium.

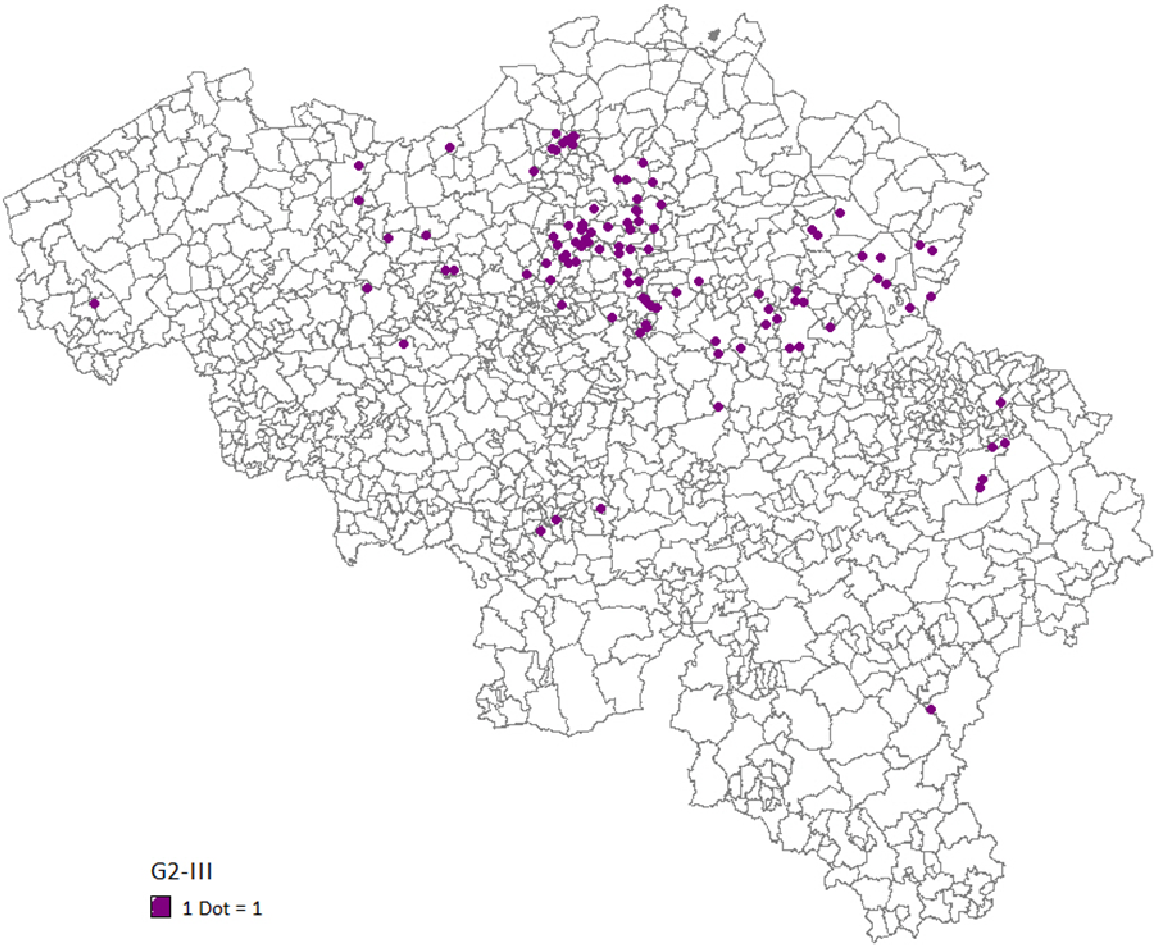

Figure 12: Geographical distribution of G2-III (purple dots). Unit: number of samples.

As for the remaining lineages (Figure 13), virtually all samples that are part of G2-IV were from the north of the country, chiefly from the province of Antwerp. The single sample that constitutes cluster G2-V is also from Antwerp.

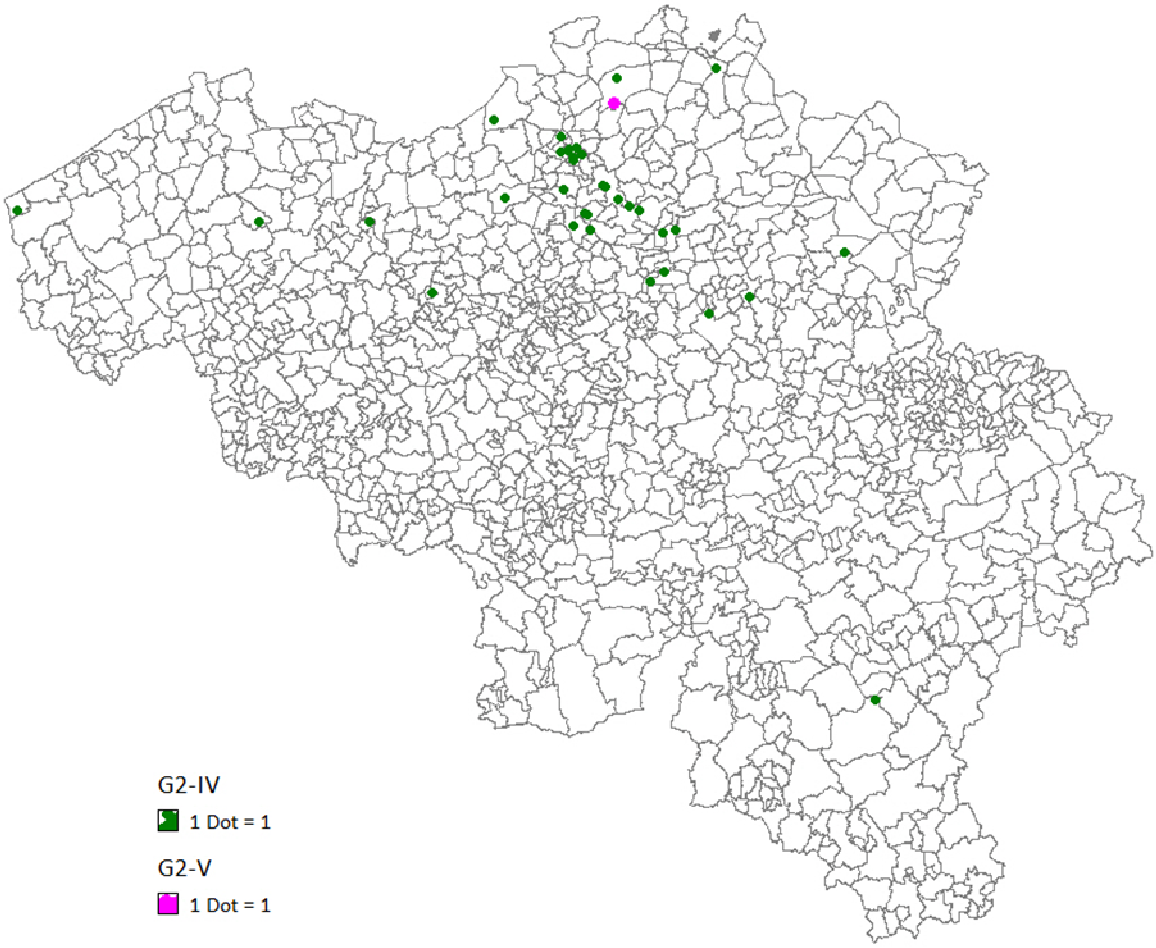

Figure 13: Geographical distribution of G2 lineages IV (green dots) and V (pink dot). Unit: number of samples.

4. DISCUSSION

The incidence of rotavirus gastroenteritis has been monitored in the Gasthuisberg University Hospital (GUH), Belgium, since 1986. Until 2006, the percentage of RV positive cases at GUH was relatively stable, with an average percentage of 19%. The two rotavirus vaccines, Rotarix® and RotaTeq®, have been commercially available in Belgium since June 2006 and June 2007, respectively, and both vaccines were quickly included in the national vaccine schedule with a governmental copayment policy, which resulted in a very high vaccine uptake (23). Due to the increasing number of vaccinated children, in the next three rotavirus seasons following vaccine introduction (2006-2007, 2007-2008 and 2008-2009) the percentage of RV positive cases at GUH dropped significantly (12.4%, 9.6% and 6.4%, respectively) (23). Consequently, in order to have a better understanding of the impact of rotavirus vaccination in Belgium, in the last three seasons (i.e. since 2007) we have been collecting stool samples from paediatric patients from all over Belgium.

The present study reports the genotype distribution of rotavirus isolated from faecal samples collected from Belgian paediatric patients in the season 2009-2010 (fourth season after vaccine introduction). The G and P genotypes were determined for the 491 positive samples, from which four samples were G- untypeable (0.8%), and seventeen samples were P-untypeable (3.5%). This genotype failure might be explained by nucleotide sequence differences between the target region of the respective genes and the primer sequences used for typing (mismatching) (3), possible low viral load of the samples, or degradation of the viral RNA after storage.

The most common HRVs worldwide are G1P[8], G2P[4], G3P[8], G4P[8], and G9P[8], with G1P[8] being the most frequent genotype combination (23, 26, 41). In our study, G2 (61.9%) was the most prevalent genotype in the season 2009-2010, whereas G1 (25.3%) was the second most prevalent (Figure 4). In this season, we also found the genotypes G3, G4, G6, G9 and G12, although in lower percentages (less than 4%). As expected, the genotypes G1, G3, G4, G9 and G12 were mostly found in association with the P[8] genotype, whereas G2 was only found associated with P[4]. Nonetheless, in this study, we found some uncommon genotype combinations (Figure 6): G1P[4], G6P[14], G9P[4], G3P[6], G12P[6], and G9P[6]. The G1P[4] and G9P[4] rotavirus strains

found in this season resulted from reassortants among common human genotypes, most likely between a G2P[4] strain and a G1P[8] or G9P[8] strains. In Belgium, the G3P[6] and G12P[6] rotavirus strains found in the current season are considered rare human genotypes. G3 and G12 human rotaviruses are usually associated with the P[8] genotype, but they can also be found in association with P[6] and, rarely, with P[9] (6, 16). The prevalence of rotaviruses with a P[6] genotype in European countries was less than 5% during the years 1996-1999 (113, 145-147). In contrast, a higher prevalence of P[6] was found in India, Bangladesh and some African countries, including Nigeria, Guinea-Bissau, Ghana and Malawi (120, 133, 148-151). Moreover, P[6] is regarded as a major porcine pathogen, reported to be present in pig herds worldwide (152). The phylogenetic analysis of P[6] strains has revealed the existence of HRV strains clustering within porcine lineages (and vice versa), suggesting complex interspecies transmission events involving human and porcine rotaviruses (153, 154). The G9P[6] strain found in the 2009-2010 rotavirus season was probably a result of this porcine-to-human interspecies transmission. Likewise, the two G6P[14] rotavirus strains found in this season were also suspected to have animal origins. G6 rotaviruses are suspected to be of bovine origin because they are frequently isolated from cattle and are only rarely encountered in humans (26, 155-159). The P[14] genotype is commonly found in rabbits, but it has also been described in goats, antelope, cattle, sheep, and lama. In the past few years, an increasing number of human P[14] rotavirus strains, mainly in combination with G6, have been described across the globe (20, 25, 160-163). We speculate that the potential animal strains found in human children in the 2009-2010 rotavirus season were due most likely to child contact with pigs (G9P[6]), rabbits, goats, sheep, or cattle (G6P[14]), at a farm or perhaps something similar. Overall, the genotype distribution analysis revealed a marked diversity among the strains collected.

As shown in Figure 7, the majority of the samples collected in the 2009-2010 rotavirus season were from the north of Belgium (Flemish Region, also called Flanders), with the higher number of samples coming from the provinces of Antwerp (43.3%), Flemish Brabant (18.2%) and East-Flanders (17.9%). Most samples appear to be coming from the north of Belgium because fewer hospitals, paediatricians and test-centres in the south of the country send their samples to our laboratory. Additionally, the geographical analysis of G-types distribution showed us that in nearly all provinces

the most predominant G-type was G2, even though we see some regional variations: G1 was occasionally more prevalent in some settings (West-Flanders, Luxembourg, Walloon Brabant, Namur), although these results were not significant due to the small number of samples from those provinces. The results were expected, since the previous analysis (Figure 4) revealed that G2 was the most prevalent genotype in the 2009-2010 season. Furthermore, Figure 7 showed us that the other genotypes (G3, G4, G6, G9 and G12) were distributed in low quantities across the country, but they were not found in unison in every province, except in Antwerp, where all different genotypes detected in the 2009-2010 season were present. In fact, G6 was only detected in Antwerp, whereas G12 was found in Antwerp and Flemish Brabant. Out of the ten Belgian provinces, G3 and G4 were found together in four of these provinces (Antwerp, Flemish Brabant, East-Flanders and Hainaut); G4 was detected in Liège as well. G9, however, was found in just three provinces (Antwerp, East-Flanders and Liège). In the remaining provinces, G1 and G2 were the only G-types detected. In short, the map analysis revealed a major gathering of different RV genotypes in the north of Belgium, specifically in Antwerp, East-Flanders and Flemish Brabant.

The data presented in Figure 8 provided us some insight in the evolution of the genotype distribution in Belgium between 2007 and 2010. Even though fluctuations in the ratio of G1, G3, G4, G9 and G12 are noticeable, we see that the relative prevalence of G2 has been constantly increasing in the last three rotavirus seasons (40.1% in 2007-2008, 47.8% in 2008-2009 and 61.9% in 2009-2010). Between 2008-2009 and 2009-2010, this increase was roughly 14%.

Although RotaTeq® has been introduced in Belgium only one year later than Rotarix®, and both have been covered by the governmental copayment policy, up until now, Rotarix® remains the vaccine with the higher uptake, most likely because of its price. The govern pays for approximately 80% of the public price of the vaccines, but the parents have to pay 10.6 € per dose. Consequently, because Rotarix® is only a two-dose vaccine, opposed to the three doses of RotaTeq®, the first vaccine ends up being less expensive (23, 92). As mentioned before, both rotavirus vaccines proved to be extremely effective, having reduced drastically the number of RV positive cases at GUH ever since their introduction in Belgium (23). However, even if Rotarix® is very effective in reducing disease caused by RV infections, this vaccine seems to offer better

protection against strains associated with P[8] than against strains associated with P[4] (137). Until 2006, G2 strains (regularly associated with P[4]) had a low prevalence, but, in the first rotavirus season after the introduction of Rotarix® in Belgium (2006-2007), G2 emerged, and was responsible for approximately one third (31.5%) of the rotavirus gastroenteritis cases at GUH (23). After a compared analysis of the data collected from all over Belgium in the last three seasons (2007-2008, 2008-2009 and 2009-2010), we confirmed this is now the fourth season since Rotarix® introduction where we see a relative increase on the prevalence of G2P[4] rotavirus strains, supporting our hypothesis that this event is probably vaccine-related. In Brazil, a study conducted shortly after the licensing of Rotarix® also reported a high prevalence of G2P[4] rotavirus infection in a vaccinated population, and linked it with the RV vaccination programme that used the Rotarix® vaccine (164). Another study conducted in a non-vaccinated population in Portugal revealed G2P[4] as the predominant RV type. However, since the population was not vaccinated, this high prevalence of G2P[4] strains was considered to be within the normal fluctuation of RV genotypes (165). Given the results described in our study, our data strongly suggest that Rotarix® might be one of the factors influencing RV genotype distribution in Belgium by exerting a selective immunologic pressure on genotypes, which might have caused the emergence of G2P[4] strains.

Phylogenetic analysis of G2P[4] strains allowed us to recognize several lineages co-circulating in Belgium in the 2009-2010 season. In the phylogenetic tree for G2 strains (Figure 9), we identified three major lineages (G2-I, G2-III and G2-IV) and two minor lineages (G2-II and G2-V). For P[4] strains (Figure 10) a similar number of clusters was found, with two major lineages (P[4]-I and P[4]-III) and four minor lineages (P[4]-II, P[4]-IV, P[4]-V, and P[4]-VI). Overall, lineages II, III and V were practically identical for both G2 and P[4]. As for the other lineages, we see some differences, but they are most likely due to reassortments among strains belonging to different lineages. It was expected that lineage P[4]-VI clustered apart from the other samples because this sample was a G9P[4] strain instead of a G2P[4].

The geographical analysis of the G2P[4] lineages (Figures 11-13) revealed no differences in the distribution pattern of the larger clusters: G2-I, G2-III and G2-IV were typically found in the north of Belgium. Since the majority of the samples collected in

our study came from the north of the country and the genotype most prevalent was G2, these results were somewhat expected. However, smaller clusters can only be found in a specific area of Belgium: G-II in Liège and G-V in Antwerp.

5. CONCLUSION

After the licensing of Rotarix® in 2006, it has been observed a relative increase in G2P[4] strains. In the investigated season (2009-2010), we saw an even further relative increase of G2P[4] (above 60%). This is now the fourth season after vaccine introduction in which an increase in the relative prevalence of G2P[4] was observed, suggesting an influence of vaccination on the RV genotype distribution. Nevertheless, we should keep in mind that this is a relative increase, and that, in absolute numbers, G2P[4] rotavirus strains are also declining, thereby reflecting the efficacy of vaccination programmes.

Our analysis demonstrated that is important to monitor genotype distribution post-vaccination, since this might be one of the factors influencing the pattern and distribution of the most prevalent rotavirus genotypes. Moreover, prospective surveillance is urgently needed because the existence of different lineages might influence future vaccine effectiveness. Further studies comparing genotype ditribution in both vaccinated and non-vaccinated populations should also be addressed.

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APPENDICES

APPENDIX A – PAGE GELS EXAMPLES

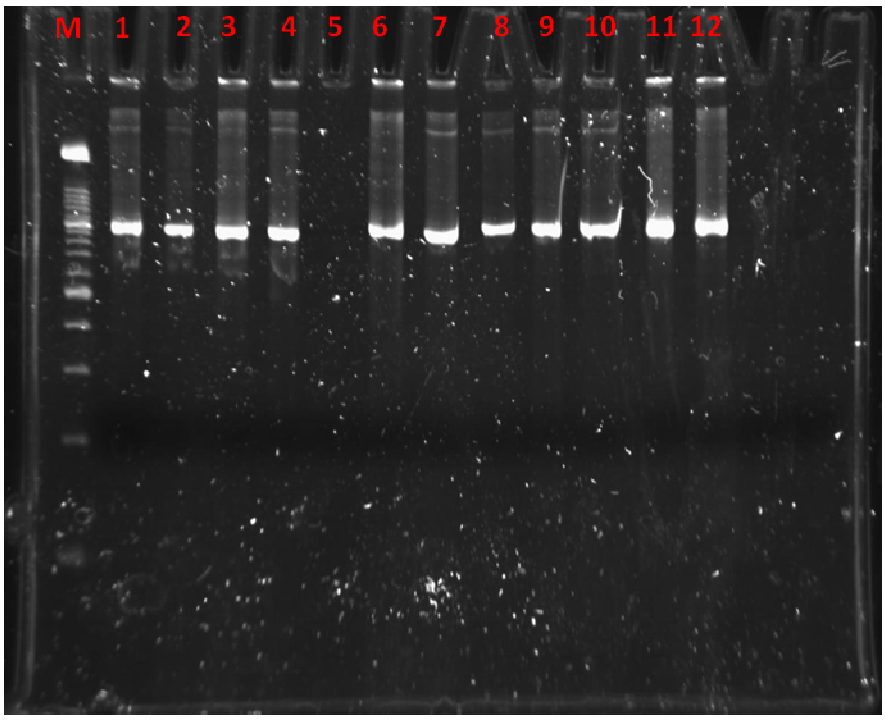

Figure 14: Polyacrylamide gel electrophoresis of twelve PCR products for the VP4 gene segment (M= Marker XIV). Eleven samples were positive (bright bands).

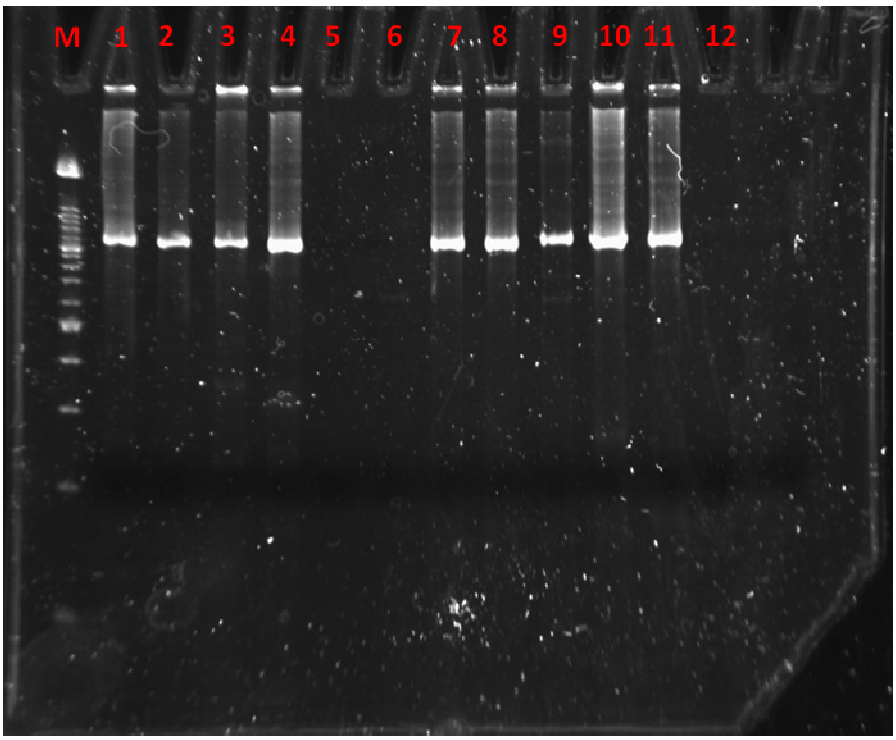

Figure 15: Polyacrylamide gel electrophoresis of twelve PCR products for the VP7 gene segment (M= Marker XIV). Nine samples were positive and three were negative.

APPENDIX B – GENE SEQUENCES

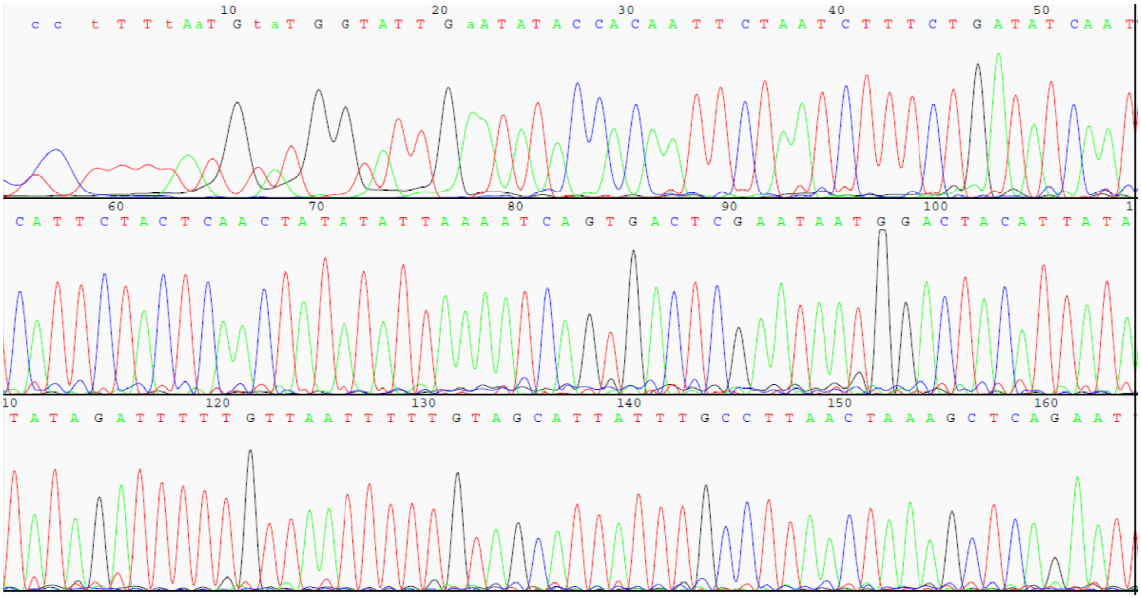

Figure 16: Example of a G1 sequence (first 163 bases).

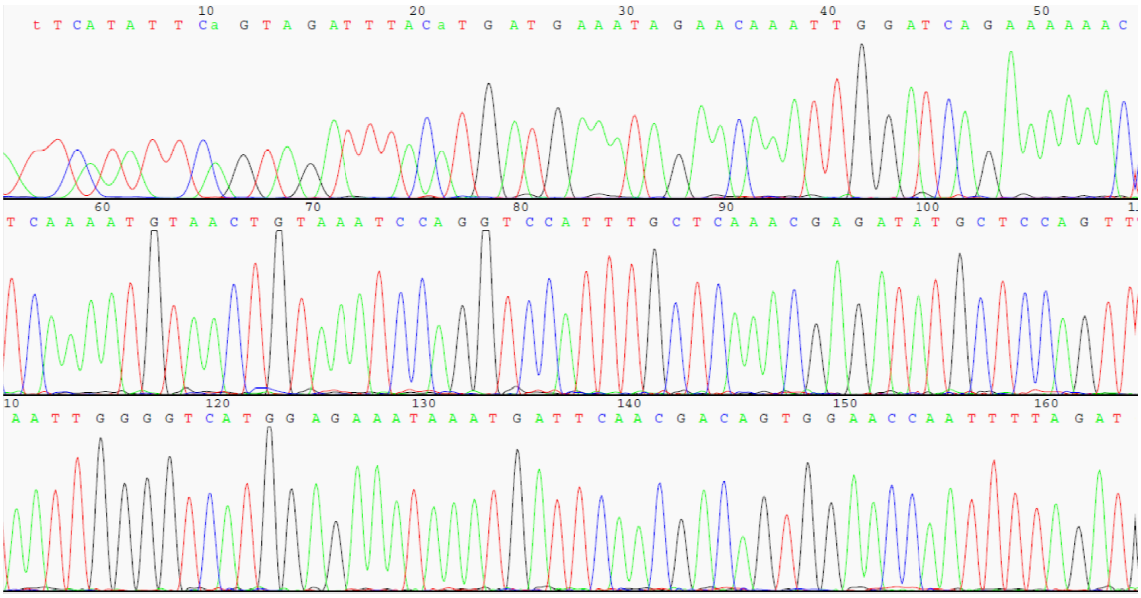

Figure 17: Example of a P[8] sequence (first 163 bases).

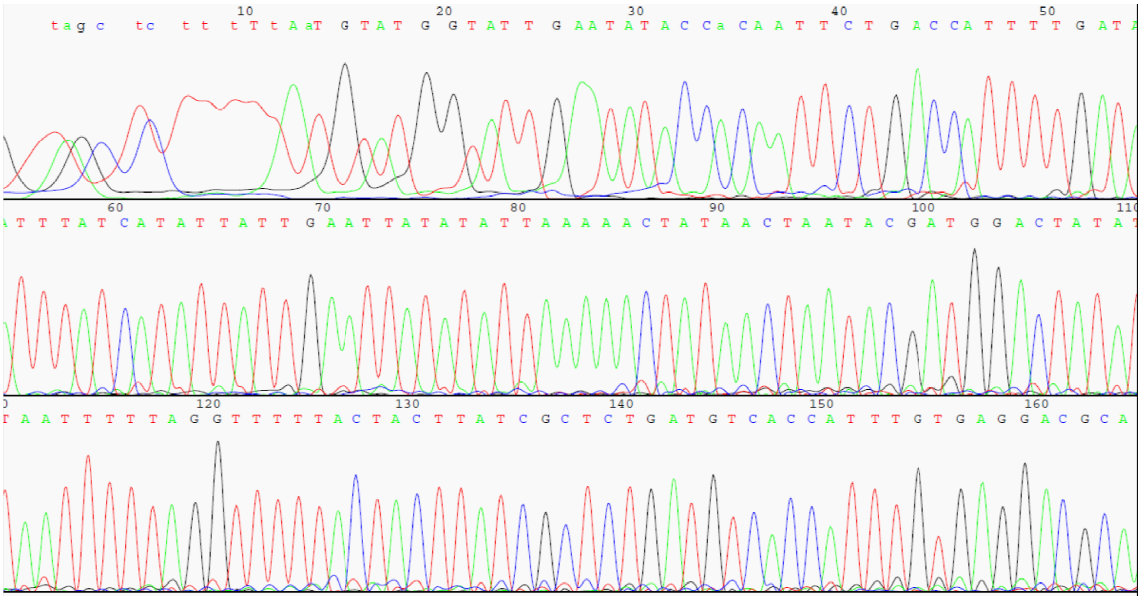

Figure 18: Example of a G2 sequence (first 164 bases).

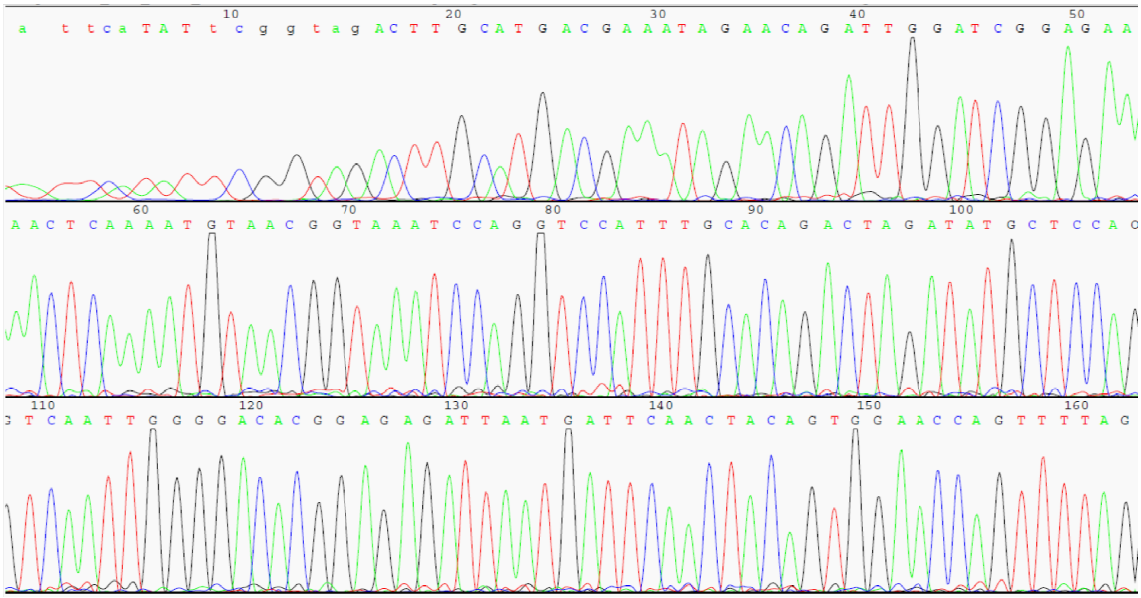

Figure 19: Example of a P[4] sequence (first 162 bases).

APPENDIX C – SEQUENCE ALIGNMENT

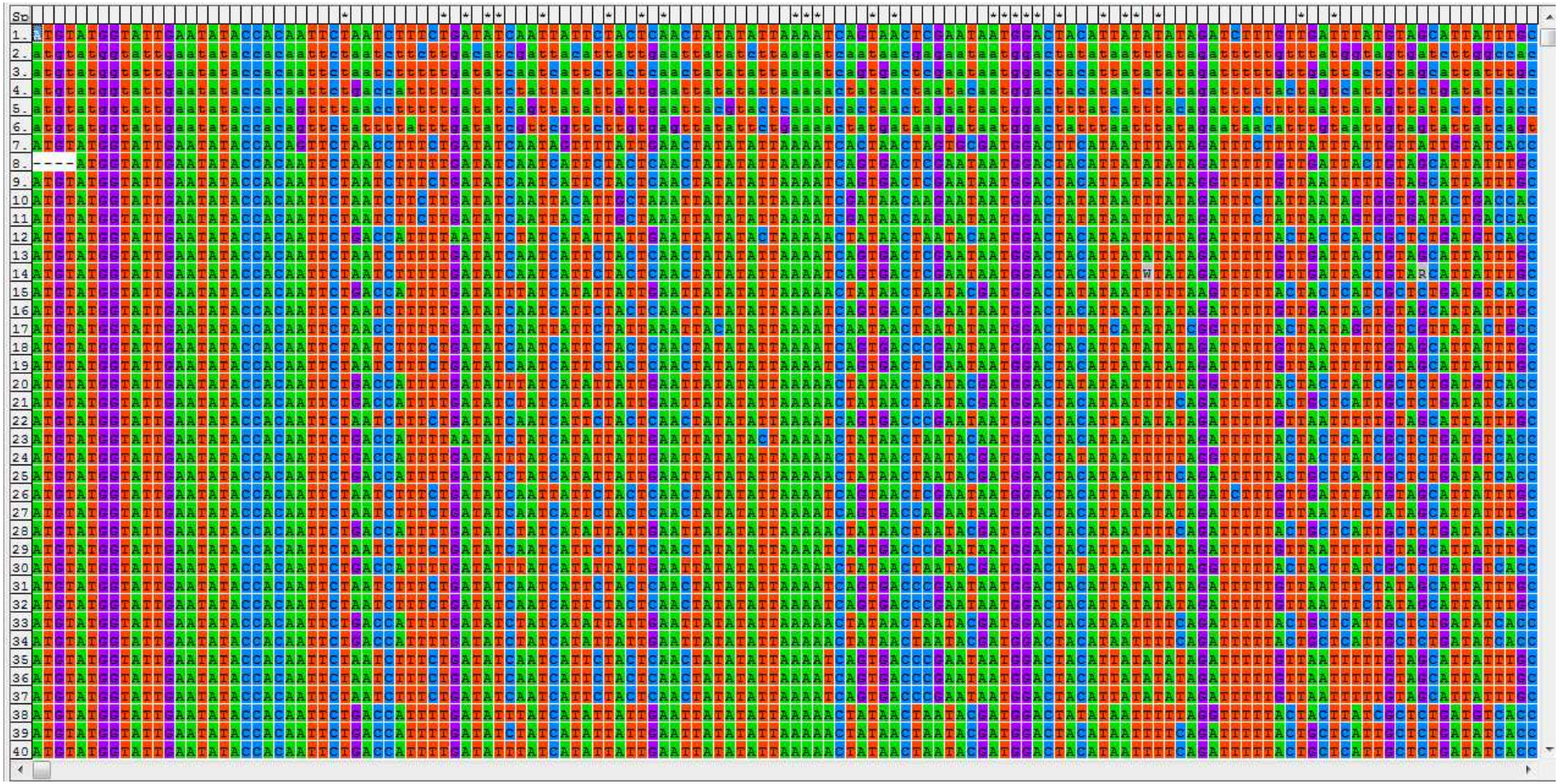

Figure 20: Example of VP7 gene sequences aligned by Mega 4.0 (and manually as well).

APPENDIX D – PHYLOGENETIC TREES

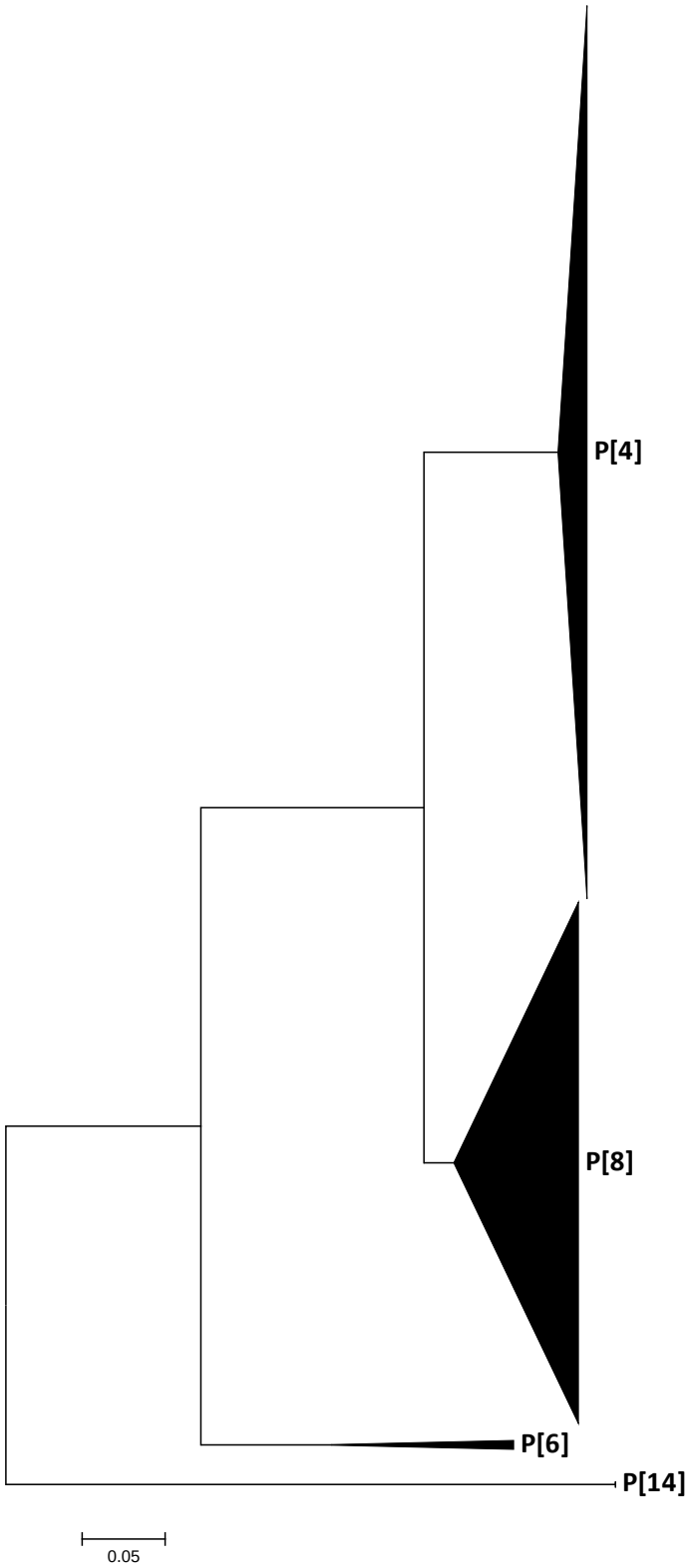

Figure 21: Neighbor-joining phylogenetic tree based on the VP4 gene sequences (2009-2010 season). Distance estimation method used: Kimura 2-parameter model.

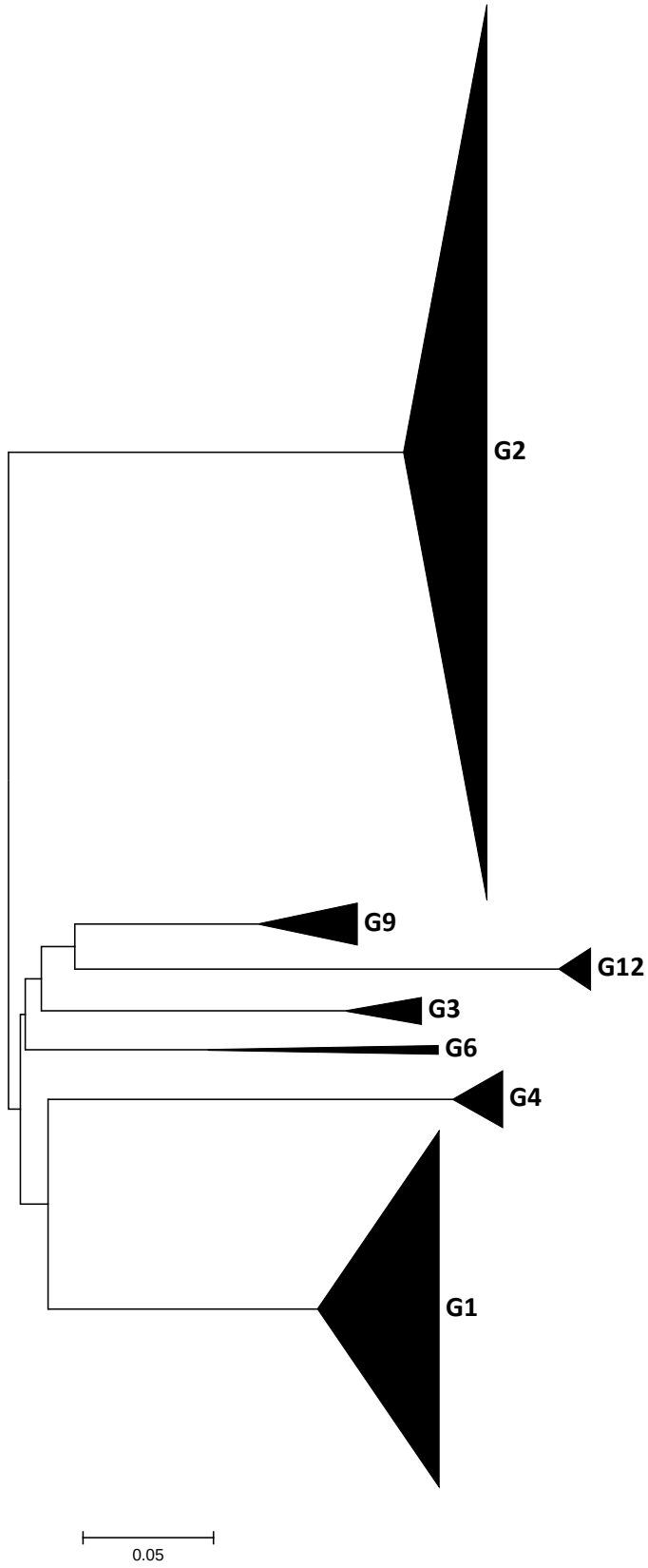

Figure 22: Neighbor-joining phylogenetic tree based on the VP7 gene sequences (2009-2010 season). Distance estimation method used: Kimura 2-parameter model.